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Investigation of enteropathogenic *Escherichia coli* adherence factors: Bundle-forming pili and intimin

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partiafulfillment of the requirements for the degree of Master of Science

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Faculty of graduate Studies and Research

The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled Investigation of enteropathogenic *Escherichia coli* adherence factors Bundle-forming pili and Initmin submitted by Johanna Marie Nemiroff in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

Initial colonization of the small intestine by Enteropathogenic *Escherichia coli* (EPEC) is mediated by bundle-forming pili (BFP). Subsequent intimate attachment is facilitated by Intimin, which binds to one or more receptors on host cells. This research investigated the effect of carbohydrate sources on regulating the expression of *bfpA*. Experiments compared *bfpA* promoter activity in a novel EPEC *Lux* operon reporter strain to that of BfpA expression in wild type EPEC. We discovered that wild type *bfpA* expression is regulated by bacterial growth rate and that *bfpA* promoter expression in the *Lux* reporter strain did not accurately reflect wild type BfpA expression.

This research also investigated whether the binding region of Intimin (Int280) possesses a functional carbohydrate recognition domain (CRD) capable of binding to the eukaryotic surface antigen Le^x. Using a solid-phase binding assay and immunoblotting we did not detect binding between purified Int280 and Le^x-BSA or related BSA-glycoconjugates.

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Abs	absorbance
A/E	attaching and effacing
AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolylphosphate
BFP	bundle-forming pili
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
CFU	colony forming unit
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
pDNA	plasmid DNA
eae	*E. coli* attaching and effacing
EAF	EPEC attachment factor
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EPEC	enteropathogenic *Escherichia coli*
esc	*E. coli* secretion
Esp	*E.coli* secreted protein
ELISA	enzyme linked immunoabsorbant assay
FBS	Fetal bovine serum
LacNAc	N-acetyl lactosamine
Ig	immunoglobulin

kDa	kilodalton
kb	kilobases
LA	localized adherence
LB	Luria broth
LEE	locus of enterocyte effacement
LeX or Le^x	Lewis X
LeY	Lewis Y
MBP	maltose-binding protein
MEM	minimal essential medium
NBT	nitroblue tetrazolium
ORFs	open reading frames
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sep	secretion of EPEC proteins
TBST	Tris buffered saline with Tween-20
Tir	translocated intimin receptor
TSB	tryptic soy broth
Tris	tris (hydroxymethyl) aminomethane
Tween	polyethylenesorbitan monolaurate
v/v	volume/volume
w/v	weight/vol

Introduction

As a permanent resident of the human gastrointestinal tract, *Escherichia coli* represents the most abundant facultative anaerobe of the normal flora, where it establishes a mutually beneficial relationship with its host. Ordinarily, *E. coli* remains a harmless species in the intestine, however in circumstances in which the gastrointestinal confines are damaged, or in an immunocompromised host, even nonpathogenic strains of *E. coli* can cause infection. Furthermore, several pathogenic strains of *E. coli* exist which have acquired the ability to cause clinical conditions such as urinary tract infections, sepsis, and a plethora of diarrheal diseases in healthy individuals (74). The first indication that certain *E. coli* strains were associated with diarrhea was evidenced in the 1940s (6, 7). Since then, research in the field of enterovirulent *E. coli* strains has gained great momentum. At least six categories, distinguished on the basis of mechanisms by which they induce diarrheal diseases are now recognized (Table 1). The categories include enterotoxigenic *E. coli* (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), enterohemorrhagic (EHEC), enteropathogenic (EPEC), and diffuse adhering (DAEC). Each group follows a common strategy in microbial exploitation of the human host; that is, colonization of a mucosal surface and evasion of host defense mechanisms (18, 74, 76). In order for enterovirulent *E. coli* to establish colonization of the intestinal mucosa, they must overcome peristalsis, and compete with the normal flora of the gut including innate *E. coli* strains, and other potential pathogens for nutrients, and attachment sites. However, the different classes of *E. coli* exhibit impressive variety in the way in which they interact with the host mucosal surface to establish colonization and localization to the target tissue. They have also developed sophisticated pathogenic

Table 1: Classes of enterovirulent *Escherichia coli*

Enterovirulent *Escherichia coli*	Site of Colonization	Major virulence Factors	Clinical Symptoms
Enterotoxigenic	small intestine	enterotoxins LT and ST, CFA	watery diarrhea, usually self-limiting
Enteropathogenic	small intestine	BFP, EspA ? Intimin	watery diarrhea, vomiting, fever
Enterohemorrhagic	large intestine	Intimin, Enterotoxins, Stx1, Stx2	bloody diarrhea, HC HUS
Enteroaggregative	adhere to small and large intestinal cells in *IVOC*	AAF/I, AAF/II EAST	watery, mucoid diarrhea excess mucus production, biofilm formation
Enteroinvasive	large intestine	enterotoxin Invasins	Shigella-like dysentery
Diffusely Adhering	not known	fimbriae, finger-like projections	potentially cause diarrhea

LT = heat labile toxin, ST = heat stable toxin, CFA = colonization factor antigen, Stx1, Stx2 = shiga toxin 1 and 2, HC = hemorrhagic colitis, HUS = hemolytic-uremic syndrome, AAF = aggregative adherence factor, EAST = enteroaggregative heat stable-like enterotoxin, IVOC = *in vitro* organ culture, BFP = bundle-forming pili, EspA = EPEC secreted protein A

strategies, including toxin production, enterocyte invasion, and gross destruction of intestinal epithelial cells, all of which contribute to the development of disease (74, 76). It is likely that *E. coli* strains capable of causing diarrhea have obtained virulence factors from enteric pathogens such as *Vibrio cholerae*, and *Shigella dysenteriae* transferred by plasmids, or lysogenic bacteriophage (27, 74). Most *E. coli* strains possess large plasmids that typically encode multiple virulence factors, and both EHEC and EPEC also encode a cluster of virulence genes on a chromosomal pathogenicity island, suggesting an earlier recombination event (27, 74).

Enteropathogenic Escherichia coli (EPEC) infection

EPEC are a well recognized causative agent of infant diarrhea, particularly in developing countries (74), with EPEC infection responsible for the deaths of several hundred thousand children worldwide annually (9). Essential for the initiation of any bacterial disease is the ability of the bacteria to localize to a niche suitable for growth and pathogenesis in the host (14). The first step in bacterial attachment is often mediated by fimbriae, or pili, which are proteinaceous structures that emanate from the bacterial surface (14). These pili can range in size from two to 50 nm in diameter, and can extend up to 20μm from the bacterial cell. Pili, which are composed of a single repeating protein subunit, may contain an additional adhesive protein at their tip, which serves to bind host cell receptors to facilitate initial attachment. Alternatively, the main structural subunit protein dispersed throughout the pilus structure may itself serve as an adhesin (14). The pilus adhesin, and host receptor combination largely dictate host, and tissue specificity, and provide a plausible explanation for why certain enteric bacteria are only able to colonize precise sections of the intestine

(14). The environmental niche in which EPEC have successfully evolved to colonize is the small intestinal epithelium, where fluid movement is great, yet competing flora is minimal relative to the large intestine (14).

Identification of Bundle forming pili (BFP)

EPEC were first noted to adhere to tissue culture cells in a distinct pattern (8, 86). The ability of EPEC to attach to cultured cells as discrete clusters of bacteria was found to be dependent on the presence of a large plasmid, of ca. 80 kb (3, 51, 63). The observed pattern of EPEC attachment *in vitro* is known as the localized adherence (LA) phenotype, and the plasmid responsible is appropriately referred to as the EPEC attachment factor (EAF) plasmid. The factor accountable for EPEC LA was first identified as long, rope like filaments extending from the bacterial surface (28). Higher magnification of the structures revealed many pilus filaments laterally aggregated to form bundles measuring 50 to 500 nm wide, and up to 20 μm long. These filaments were termed bundle-forming pili (BFP) (28), and bundles produced by individual bacteria appeared to be intertwined mediating inter-bacterial interactions thus forming a bacterial network, or microcolony. BFP are composed of a polymer of an individual protein subunit, of molecular mass 19.5 kDa (28). The EAF plasmid encoded *bfpA* gene directs the synthesis of this major structural protein termed bundlin, or pilin (12), a member of the type IV pilus protein family (12, 87). Bundlin is first expressed as a prepilin protein, possessing an N-terminal leader sequence cleaved by a prepilin peptidase. The mature protein possesses a hydrophobic domain at its N-terminus, and two cysteine residues that form a disulfide bond near the C-terminus,

which is consistent with other type IV pili produced by a variety of bacterial pathogens (5, 40, 92).

Assembly of BFP

Biogenesis of EPEC BFP is a complex process, which closely resembles that of the TCP (toxin-co-regulated pilus) of *Vibrio cholerae* (49, 88, 91). An approximately 11 kb region of EAF plasmid DNA termed the *bfp* gene cluster was found to contain 12 to 14 open reading frames (ORFs), which function to direct the synthesis of BFP filaments (88, 91). When the *bfp* gene cluster is cotransformed with *bfpT*, a positive regulator of *bfpA* located on the EAF plasmid, but outside the *bfp* gene cluster, into laboratory strains of *E. coli*, BFP production, and the LA phenotype are attained (91). The ORFs in the *bfp* gene cluster were found to be similar to accessory proteins involved in type IV pilus biosynthesis and assembly, protein secretion, and DNA uptake mechanisms. As previously mentioned, the first gene of the cluster, *bfpA* encodes pilin, the repeating subunit comprising the BFP filament. *bfpP* encodes the prepilin peptidase, which cleaves the N-terminal leader sequence (111). An outer membrane lipoprotein encoded by the second gene in the cluster, *bfpB*, is involved in pilus biogenesis after signal peptidase cleavage (88). The sixth and eighth genes in the *bfp* gene cluster are *bfpD*, and *bfpF* respectively, which both encode putative nucleotide binding proteins thought to provide energy for BFP biosynthesis (4, 88, 91). A mutation in the putative nucleotide-binding domain of *bfpD* results in no BFP expression, and produces a phenotype that is both non-aggregative, and LA negative (4), indicating that BfpD is required for pilus biogenesis. When a similar mutation is introduced into the putative nucleotide-binding domain of *bfpF*, the mutant expressed BFP,

and was more adherent to Hep-2 cells than the wild type parent strain (1, 4). BfpF is also involved in facilitating the dissociation of bacteria from aggregated microcolonies, which may be important in the ultimate strategy of EPEC pathogenesis. Whereas wild type bacteria form autoaggregates *in vitro* that disperse over time, *bfpF* mutants failed to disaggregate (4). This occurrence was subsequently demonstrated by Knutton et al. (55), who also reported that BfpF are involved in generating a dramatic change in the quaternary structure of BFP from thin to thick bundles, thereby disrupting inter-bacterial interactions, and leading to dissociation of microcolonies. The predicted amino acid sequence of BfpF shares 32% identity to that of the PilT protein of *Pseudamonous aeruginosa*, which powers this organism's twitching motility (88, 91, 108). It has been suggested that BfpF of EPEC may play a role in pilus retraction, and that this could further contribute to disaggregation of bacterial microcolonies, however no direct evidence has been put forth to support this hypothesis (1). There are many other genes present in the *bfp* gene cluster, many of which likely function to remove bundlin subunits from the bacterial membrane and assemble them into pili (2). Also required for the successful assembly of BFP is the chromosomal *dsbA* gene. Tn*phoA* insertion mutants defective in LA were shown to have insertions in the EPEC chromosome, in addition to the plasmid encoded *bfpA* gene (13). The chromosomal Tn*phoA* insertions were later mapped to the chromosomal *dsbA* gene (110). While the *dsbA* gene product, a soluble periplasmic oxidoreductase, is not required for transcription of *bfpA*, it is required for disulfide bond formation between two cysteine residues at the C-terminal end of bundlin (110). Thus the C-terminal end of bundlin is exposed to the periplasmic space during pilus processing. DsbA activity is not dependent on signal peptidase cleavage of the N-terminal leader of prepilin. More accurately, prepilin exists as

a cytoplasmic transmembrane protein accessible to the cytoplasmic BfpP signal peptidase, and the periplasmic DsbA enzyme simultaneously during BFP assembly (11, 110).

Regulation of BFP expression

BFP expression is not constitutive rather it is regulated at the transcriptional level by a variety of environmental signals. *In vitro* induction of *bfpA* expression can be achieved by transferring 37°C stationary phase EPEC to Dulbecco's modified Eagle's medium (DMEM) containing 0.45% glucose and growing the bacteria for 30-60 minutes (104). Under these conditions, expression of the BFP subunit corresponds to the autoaggregation and LA phenotypes, and did not require epithelial cells, or their products (104). To further investigate specific stimuli that might control *bfpA* expression, the promoter region of *bfpA* was cloned into a multicopy number vector upstream of a promoterless *cat* gene. This construct was then transformed into wild type EPEC, and used to determine the effects of specific growth conditions on *bfpA* expression by monitoring CAT activity (78). Maximal expression from the *bfpA* promoter was shown to occur during the exponential phase of growth, at 37-38°C, under conditions of high calcium, and low ammonium ion concentrations (78). Carbon source has also been shown to be an important regulator of *bfpA* expression as EPEC grown in DMEM supplemented with glucose, but not galactose expressed BfpA (101). In addition to these stimuli, expression of *bfpA* also requires the presence of a plasmid encoded activating complex, *bfpTVW* (44, 78, 98). The *bfpTVW* gene cluster, which resides on the EAF plasmid downstream of the *bfp* gene cluster, is allelic with the *perABC* genes in the same plasmid (30). BfpT encodes an AraC-like activator protein that activates *bfpA* transcription (98). It is plausible that the factors shown to induce

bfpA expression *in vitro* could represent the environmental conditions encountered by EPEC upon ingestion and passage into the small intestine, thus contributing to the establishment of colonization (78).

The role of BFP in the infection process

The role of BFP in infection remains a controversial topic in the field of EPEC pathogenesis. Well documented is the fact that in tissue culture media EPEC autoaggregate into microcolonies (4, 104). Such aggregates may in turn act as infectious entities that promote direct contact of the bacteria with the epithelial cell surface (14). Indeed one robust model for EPEC pathogenesis has been proposed in which the first stage of the infection process involves non-intimate attachment of EPEC to epithelial cells as a result of BFP-mediated interbacterial interactions, and microcolony formation (12). One study focused on *bfpA* expression supports this model, and proposes that microcolony formation is likely the first step in the EPEC pathogenic process (78). Moreover, small bowel biopsies of EPEC infected children reveal discrete microcolonies of bacteria attached to mucus membranes (83, 84). It is possible that BfpA may serve as the adhesin itself, acting as the ligand for host cell receptors, although no direct evidence for this concept exists. Alternatively, BFP mediated microcolonies may facilitate the interaction of a yet unidentified EPEC adhesion factor with host cell receptors to establish colonization (14, 37) (Figure 1A). It is interesting to note that BFP-specific antiserum, when added in combination with wild type EPEC to Hep-2 cell monolayers, both reduced the number of bacteria per microcolony, and decreased the percentage of LA (28). BFP have also recently been shown to elicit antibody responses in natural infections, and to a lesser degree in

experimentally infected adults (65, 67, 75). While a BFP receptor has not yet been identified, a proposed synthetic BfpA receptor analogue pre-incubated with EPEC prior to addition of the bacteria to Hep-2 cell monolayers showed that both LA and *bfpA* expression were reduced (102). This report indicates that microcolony formation and subsequent dispersal may be important in EPEC pathogenesis. Certainly BfpF-mediated microcolony disaggregation after LA, likely plays a role in EPEC infection. BfpF mutants fail to promote dispersal of bacterial aggregates and were less virulent in human volunteers (1, 4, 55). It is probable that BfpF is required for aggregate dispersal thereby allowing EPEC to disseminate to other sites along the small intestinal epithelium (49, 81). In contrast to EPEC, *V. cholerae* lack BfpF, and remain locally concentrated throughout the infection process (49). A second model of EPEC pathogenesis utilizing *in vitro* organ culture (IVOC) is consistent with the suggestion that dispersal of EPEC microcolonies facilitates spread of the organism (37). This model, which may more closely represent *in vivo* conditions than typical tissue culture model systems, proposes that BFP are not involved in the initial stages of colonization rather are involved in later stages of infection. Here, they function to establish three-dimensional microcolonies that lift away from the epithelium thus promoting spread to other intestinal sites. This model also suggests that initial attachment requires an adhesin other than BfpA that awaits recognition. Yet, interesting to note is the fact that Enterohemorrhagic *Escherichia coli* (EHEC), a human pathogen closely related to EPEC, does not produce BFP, and infects the colon rather than the small intestine. This knowledge suggests that BFP may indeed play a role in localizing EPEC to the small intestine in order to establish initial attachment (100).

Figure 1: EPEC colonization model

(Adapted from Hicks *et al.* 1998, and Donnenberg *et al.* 1992)

A. Initial non-intimate attachment to small intestinal epithelial cells mediated by BFP, EspA (described later, see EPEC secreted proteins (Esps)), or a yet unidentified adhesin.

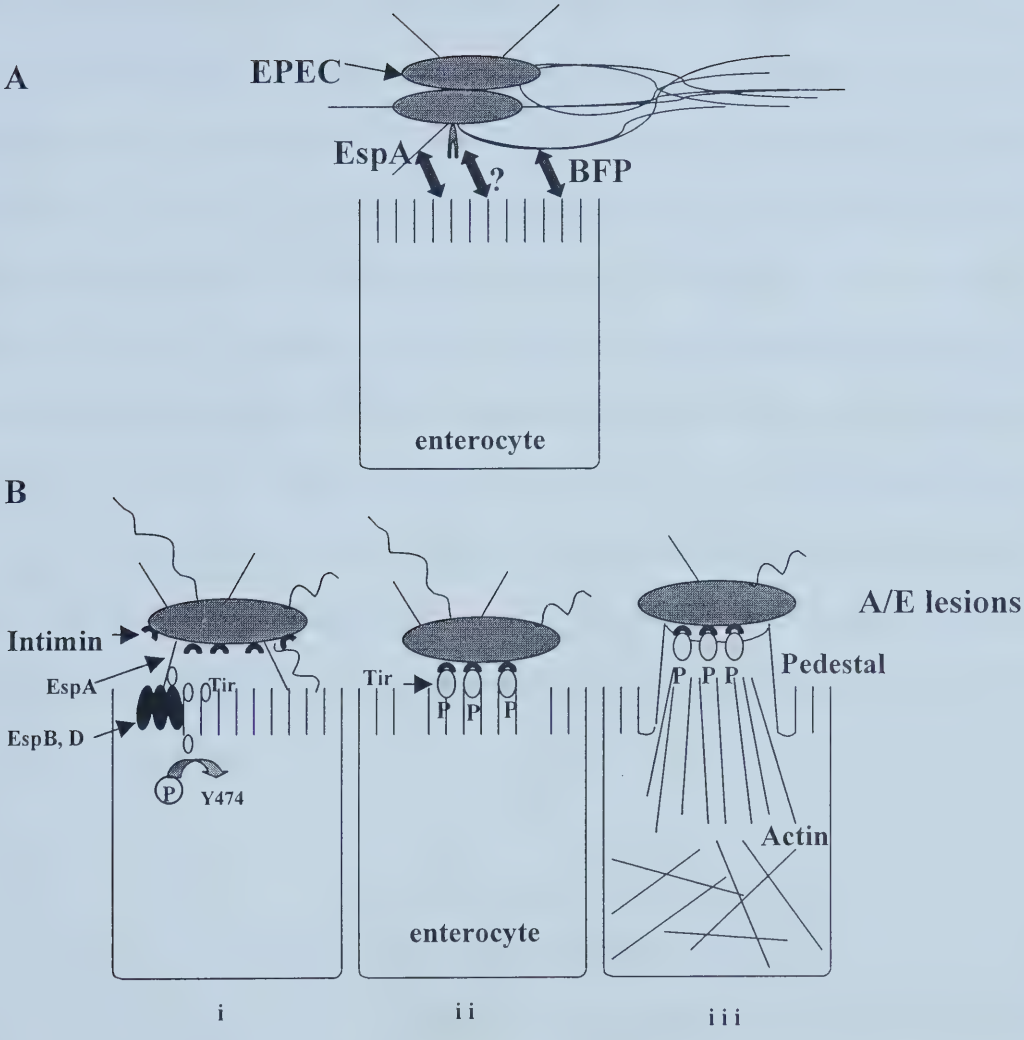
B. Initial adherence facilitates activation of the Type III secretion pathway.

i) EPEC secreted proteins Tir and EspB are translocated to the host cell initiating Tir-tyrosine phosphorylation (P) and insertion of Tir into the host cell plasma membrane. EspB and D likely function together, forming a pore-complex, which mediates the translocation of Tir and other effector proteins across the host cell membrane.

ii) EPEC adhesin Intimin binds to phosphorylated Tir on the host cell surface bringing bacteria into closer contact with the host cell. Defacement of epithelial cell microvilli begins as does signal transduction events between EPEC and the host cell, which is mediated by Intimin-Tir interaction.

iii) Cytoplasmic re-arrangements including the accumulation of polymerized actin and other cytoskeletal proteins at the site beneath attached bacteria leads to pedestal formation and characteristic A/E lesions. Additionally, BFP and EspA production is down-regulated.

iv) While not shown, EPEC may spread and colonize other sites along the intestine by way of three- dimentional microcolony formation, which lifts bacteria away from the epithelial cell surface, by way of BfpF-mediated microcolony dispersal, or via transport by dynamic pedestal movement.


A
EPEC
EspA
?
BFP
enterocyte
B
Intimin
EspA
Tir
EspB, D
P
Y474
Tir
P P P
A/E lesions
P P P
Pedestal
Actin
enterocyte
i
ii
iii

Attaching and Effacing (A/E) pathology

Undoubtedly, the most distinguishing feature of EPEC infection is the production of distinctive intestinal lesions that occur in both human and experimental animal models, and can be reproduced in cell culture (43, 72, 95, 99). Attaching and effacing (A/E) lesions are characterized by the destruction of epithelial cell microvilli accompanied by intimate attachment of bacteria. At the site of attachment, bacteria appear to be cupped by a pedestal structure that can extend many micrometers from the host cell surface (72). During A/E lesion formation a series of host cell signal transduction events and cytoskeletal rearrangements take place, resulting in the recruitment of actin and other cytoskeletal proteins, as well as signaling proteins to sites beneath tightly adhered bacteria (19, 33, 56, 74). The observation that A/E lesions contain high concentrations of polymerized actin led to the development of the fluorescent actin staining (FAS) test; a diagnostic test for EPEC and other bacteria capable of causing A/E lesions such as EHEC (56).

Locus of enterocyte effacment (LEE)

The locus of enterocyte effacment, or LEE, is a pathogenicity island of ca. 35 kb found within the chromosome of EPEC, and other A/E forming pathogens (9, 22, 74, 81). The LEE pathogenicity island is named such because it contains all of the genes necessary for A/E lesion formation (Figure 2). Characteristic of pathogenicity islands, the G+C content of LEE is 38.4%, compared to 50% of the nonpathogenic *E. coli* K-12 chromosome,

suggesting that LEE was acquired from a foreign source and subsequently inserted into EPEC's chromosome (100). There is likely a common method of A/E lesion formation as DNA sequences highly homologous to EPEC LEE have been found in other A/E lesion forming pathogens such as EHEC, the mouse pathogen *Citrobacter rodentium* as well as rabbit, dog and pig EPEC equivalents (9, 32). Introduction of the cloned LEE of EPEC (Figure 2) into a nonpathogenic *E. coli* strain bestowed the ability to form A/E lesions demonstrating that genes encoded by LEE are necessary and sufficient for A/E lesion formation (68). EPEC LEE contains at least 41 ORFs, arranged into five polycistronic operons (15, 71), and can be divided into three functional domains. The central region contains the *eae* and *tir* genes, which encode the proteins Intimin and Tir (translocated intimin receptor) respectively (15, 32, 81). These proteins are essential for intimate adherence of EPEC to host cells, a process that is discussed in detail below. Upstream of the *eae* region are a series of genes encoding the structural components of a Type III secretion apparatus; a sophisticated protein secretion mechanism used to translocate bacterial effector proteins directly to the host cell in which they exert their effects (25, 42, 109). Genes encoding proteins comprising the type III secretion apparatus are referred to as *esc* genes (for *E. coli* secretion), or *sep* genes (for secretion of *E. coli* proteins) (15). Downstream of the *eae* domain of LEE are *esp* genes encoding the secreted effector proteins (EPEC secreted proteins) (15). The function served by LEE in facilitating the development of A/E lesions is multifaceted, and the exact temporal sequence of events is not certain. Signal transduction, bacterial protein translocation, and intimate attachment are important events occurring between EPEC and the host epithelial cell, all of which

contribute to A/E lesion formation and EPEC pathogenesis. Each element is discussed below.

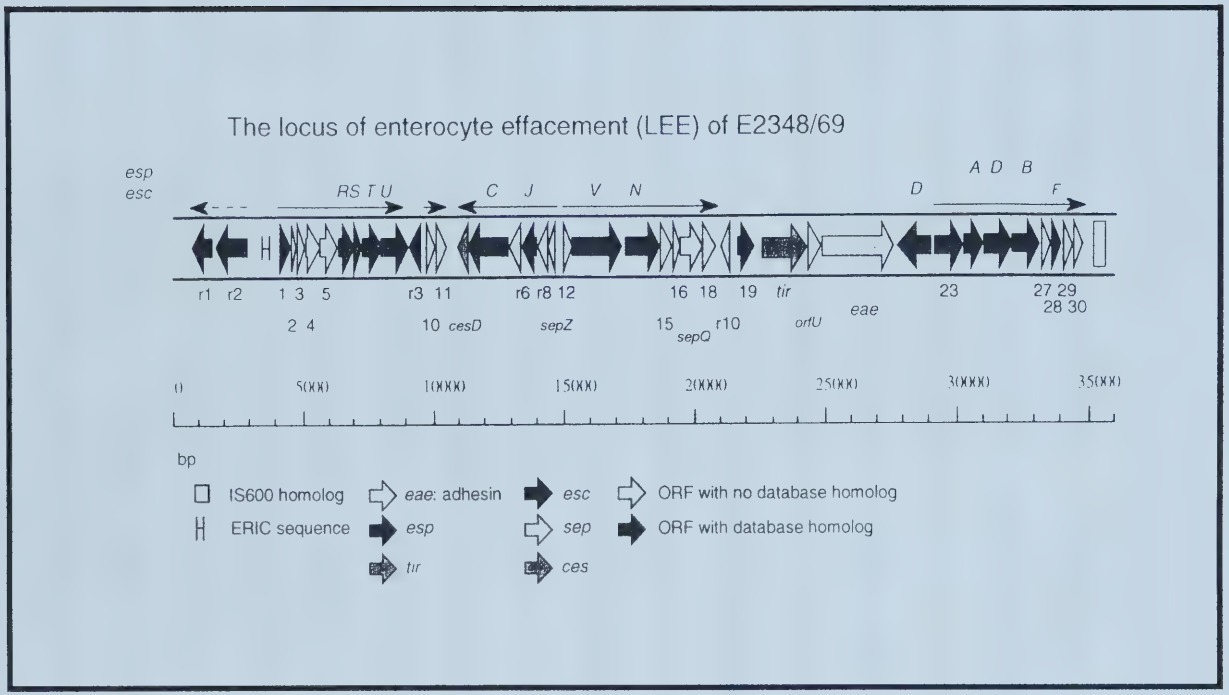
EPEC type III protein secretion apparatus

Surprisingly little information regarding the structural machinery utilized for the secretion of EPEC effector proteins is available considering the critical nature of secreted proteins including Tir for A/E lesion formation and full virulence. Six genes, *escU,C,V,N* and *sepQ,Z* were shown to be required for protein secretion by EPEC (81). Some proteins of the type III secretion apparatus are homologous to proteins implicated in the assembly of bacterial flagella (25). A structural component of the type III secretion system in *Salmonella typhimurium* was recently observed by electron microscopy to closely resemble the flagellar basal body (59). The *S. typhimurium* structure contains a long needle like portion sticking out from the surface of the cell, and a cylindrical base that anchors the complex to the inner and outer membranes (59). In EPEC it is possible that a similar type III secretion structure exists, with EscC suggested to be an outer membrane anchor protein based on homologies to YscC of *Yersinia enterocolitica* (58). It is conceivable that type III secreted proteins traverse the inner membrane, periplasm, and outer membrane by means of the hollow cylindrical channel, and translocate to the host cell by means of long projections from the exterior of the cell (25, 54). In EPEC, EscN, a putative ATPase (41), potentially supplies the energy for this process.

Figure 2: Locus of enterocyte effacement (LEE) of E2348/69

(Elliot, S. J. *et al*. 1998)

LEE contains all of the genes necessary for A/E lesion formation. The *eae* and *tir* genes encode the adhesin Intimin and its translocated intimin receptor (Tir) respectively. Upstream of these central genes are the *sep*, or *esc* genes, which encode components of the Type III secretion apparatus. Downstream of *eae* and *tir* are the genes encoding for EPEC secreted proteins, or *esp* genes. LEE also contains a homologue of IS600 (Insertion Sequence 600), a transposable element suggesting a possible means of LEE acquisition in EPEC. Lee also contains an ERIC sequence (enterobacterial repeat intergenic consensus) whose function is not known. The complete nucleotide sequence of LEE is available (accession AF022236).


The locus of enterocyte effacement (LEE) of E2348/69
esp
esc
RS T U
C J V N
D
A D B
F
H
r1 r2 1 3 5 r3 11 r6 r8 12 16 18 19 tir 23 27 29 28 30
2 4 10 cesD sepZ 15 sepQ r10 orfU eae
0 5(XX) 10(XXX) 15(XX) 20(XXX) 25(XX) 30(XXX) 35(XX)
bp
IS600 homolog
ERIC sequence
eae: adhesin
esp
tir
esc
sep
ces
ORF with no database homolog
ORF with database homolog

EPEC secreted proteins (Esps)

Upon initial non-intimate attachment, EPEC secretes a number of proteins (Esps) to the extracellular environment by means of the type III secretion system that are essential in establishing intimate adherence and A/E lesions. These proteins are either delivered (translocated) to the host cell where they alter specific host cell activities, or may function coordinately in the secretion process itself (25). The main secreted proteins involved in A/E lesion formation include EspA, EspB, EspD, and Tir. Deletion of these proteins results in loss of the A/E pathology (32, 48, 60). EspA is a structural protein thought to form the main component of a filamentous attachment/delivery system (54). EspA filaments are reported to be expressed prior to host cell contact, and form a direct link between EPEC and the host cell surface (54). Furthermore, EspA filaments facilitate the translocation of other proteins such as EspB, and Tir to the host cell (48, 54), and are not produced after intimate attachment and A/E lesion formation has occurred. *espA*$^{-}$ mutants are capable of EspB secretion, but not translocation (60). The interaction between EspA filaments and the eukaryotic cell is weak, and the possibility that other adhesins are involved in initial non-intimate attachment, cannot be excluded (53, 77). EspB is found in both the cytosolic and membrane fractions of infected host cells (53, 109). EspB likely has a multifunctional role as it appears to be required for the translocation process itself (109). Putative EspB functions include pore formation in the host cell plasma membrane permitting translocation of other proteins, and acting as an effector protein involved in disrupting the host cell cytoskeletal matrix (32, 96, 97). EspD is thought to form a pore in the host cell plasma membrane in complex with EspB (32, 105). Other recently identified EPEC

secreted proteins include EspF (69), EspG (16), and EspC (70, 90), however the exact function of these proteins is still under investigation.

Intimin, Tir, and intimate adherence

EPEC intimate adherence is mediated by Intimin, a 94 kDa outer membrane adhesin encoded by the *eae* gene of LEE, binding to its receptor Tir (translocated intimin receptor) on the surface of host cells. The intimin receptor was originally thought to be a host protein (82), however it has since been discovered to be an EPEC encoded protein, that is translocated to the host cell by way of the type III secretion apparatus (48). Upon translocation and insertion into the host cell plasma membrane Tir undergoes phosphorylation and other host mediated modifications presenting itself as a 90 kDa protein when analyzed by SDS-PAGE (47, 48). Tir represents the first example of a pathogen injecting its own receptor into a eukaryotic host cell (48, 100). Tir spans the host plasma membrane twice displaying an extracellular loop which contains the intimin binding domain, as well as cytoplasmic amino and carboxyl termini (9). Intimin, which is anchored in the EPEC outer membrane binds and clusters the phosphorylated form of Tir in the host cell plasma membrane leading to signal transduction events, intimate attachment, and characteristic pedestal formation (31, 32) (Figure 1B). Intracellular C-terminal Tir phosphorylation on tyrosine (Y474) is required for actin accumulation at the site of bacterial attachment (47). Yet the presence of a tyrosine protein kinase inhibitor does not inhibit A/E lesions (79), consequently the significance of Tir phosphorylation remains to be determined. In addition to actin, many other cytoskeletal proteins are

recruited to the site of EPEC attachment upon Tir translocation (33). One such protein, α-actinin, interacts directly with Tir at the N-terminus independently of Tir phosphorylation (31). In accordance with A/E formation EPEC induces many host cell-signalling cascades that are likely mediated by Esps (32, 74, 81, 100). Studies involving the interaction between intimin and Tir demonstrate that the binding domain of intimin resides in the C-terminal 280 amino acids (Int280) (21, 24). In the absence of Tir, or prior exposure to secreted proteins, Int280 is still able to bind to host cells (21, 23, 24, 35), suggesting the existence of a second intimin receptor of eukaryotic origin. Furthermore, an EPEC strain deficient in EspA filament assembly and EspB and Tir translocation, formed A/E lesions indistinguishable from wild type, albeit at a much slower rate (10). Nevertheless this result supports the theory of a second intimin receptor. While Int280 has been reported to bind to β1-integrins (20), they are not expressed on the apical surface of enterocytes, and the relevance of this interaction has been questioned (64).

Regulation of A/E virulence factor expression

Similar to BFP expression, A/E virulence factor expression is regulated by environmental conditions such as growth phase, temperature, and other conditions that represent the intestinal environment (46, 80). Additionally, A/E expression is induced in response to *de novo* protein synthesis upon EPEC contact with the host cell (80). As previously discussed, the *perABC/bfpTVW* locus of the EAF plasmid encodes a regulatory complex that activates expression of plasmid-encoded *bfpA* for BFP production. The *per* locus is actually a global regulator that also functions to activate expression of LEE-encoded A/E virulence genes like *eae* and *espB* in trans (30, 98). Intimin expression has been shown to decrease in response to host cell factors after A/E lesions have formed (52), and is also slightly

reduced when EPEC are incubated with the synthetic glycoconjugate LeX-BSA (102). Consistent with these findings is the observation that EspA filaments are transiently expressed prior to A/E lesion formation (54). It therefore seems that EPEC responds to host cell signals to down regulate A/E virulence genes when their expression is no longer required. EPEC may also respond to bacterial factors that are produced when bacteria grow in high density (or a quorum) in order to coordinate virulence factor expression (89). Regulation of LEE-encoded genes in response to quorum sensing may signal to EPEC when they have reached their proper niche to activate A/E virulence genes.

Premise for proposed research

Research initiatives involving two EPEC attachment factors BFP and intimin were considered independently. Previously Vanmaele *et al.* reported that BfpA expression and EPEC LA was reduced when the organisms were grown in DMEM containing galactose as the carbon source (101). To further investigate carbon source regulation of BFP expression, the *bfp* operon promoter was cloned into pCS26 upstream of the promoterless *lux* operon from *Photorhabdus luminescens* (Dr. M. Surette, University of Calgary, personal communication, Figure 3). The resulting *lux* reporter plasmid pMS420 was then transformed into wild type EPEC E2348/69. Transformants (E2348/69[pMS420]) were subsequently grown in the presence of galactose and additional carbon sources. Dr. Surette could then monitor *bfp* promoter activity by measuring light produced by E2348/69(pMS420) grown in the presence of the different carbon sources. High, medium, and low expression activity from the *bfp* operon promoter was observed when the *lux* reporter strain was grown in glucose, arabinose, or glucosamine; galactose; and gluconate

respectively. The luminescence produced by E2348/69(pMS420) in these experiments appeared to correlate with the previous finding of Vanmaele *et al.* That is, *bfpA* expression is high when wild type EPEC is grown in the presence of glucose, and decreased when grown in DMEM containing galactose (101). I therefore reasoned that the endogenous *bfpA* promoter would respond in a similar manner as the cloned *bfp* operon promoter when wild type EPEC are grown in the presence of the various carbon sources. To test this hypothesis, I grew wild type EPEC in the presence of the same carbon sources used to grow the *lux* reporter strain and monitored *bfpA* expression levels and the LA phenotype. This study represents the first attempt to establish a correlation between the *bfp* expression levels observed in the *lux* reporter strain to the actual *bfp* expression and LA phenotype of wild type EPEC. Intimin is an EPEC attachment factor that binds to Tir on host cell surfaces. However, the C-terminal portion of Intimin (Int280) can bind to uninfected host cells in the absence of Tir, which has led to the speculation that a second intimin receptor of eukaryotic origin exists. C-type lectins are a family of proteins that recognize cell surface carbohydrates (107). Int280 possesses a C-type lectin domain that has been shown to bind to β1-integrins (20) although the physiological significance of this binding has not been demonstrated. In accordance with Frankel *et al.*, we also suspected that Int280 recognizes a eukaryotic carbohydrate. In the *C. rodentium* mouse infection model, intimin from *C. rodentium* or EPEC induced colonic epithelial hyperplasia concurrent with a strong T_H1 cell response (38). Moreover bacterial viability was not required for these events, and a mutant strain expressing an Intimin altered in the putative lectin domain failed to elicit the same response (39). These reports further support the theory that intimin binding to a host receptor is necessary to elicit the host immune response. Interestingly,

intimin expression is reduced when EPEC is pre-incubated with the Le^X-BSA glycoconjugate (102), consistent with the observation that intimin expression is down regulated after A/E lesion formation (52). These studies combined with evidence that Int280 has a C-type lectin domain, can bind to uninfected host cells, and can bind to host cells in the absence of Tir, prompted further investigation of Int280. A solid phase Int280 binding assay was designed to test the hypothesis that Int280 is capable of recognizing a eukaryotic surface carbohydrate such as Le^X.

Chapter 2

Carbon source regulation of *bfpA* expression

Bfp expression studies typically involve growing EPEC under a variety of experimental conditions and simultaneously monitoring *bfpA* expression levels and LA to tissue culture cells. The *bfpA* promoter region has previously been cloned into a multicopy number vector upstream of a promoterless *cat* gene (78). This construct was transformed into wild type EPEC and used to determine factors regulating *bfpA* expression by measuring CAT activity. This system was not used to investigate the effect of carbon source on regulation of *bfpA* expression in EPEC. Recently, the *bfpA* promoter region was cloned into a low copy number plasmid, pCS26 upstream of the promoterless *Photorhabdus luminescens lux* (light production) operon (Figure 3). The resulting construct, pMS420, was transformed into wild type EPEC and used to investigate carbon source regulation of *bfpA* expression by measuring light production (Dr. Surette, University of Calgary, personal communication). Since pMS420 is a low copy number plasmid, its presence in E2348/69 is not expected to alter *bfpA* expression from the wild type EAF plasmid. Correspondingly, the *bfpTVW* regulatory element encoded by the wild type plasmid is expected to activate endogenous and cloned *bfpA* expression. The rationale for constructing the EPEC Lux reporter strain was to facilitate monitoring expression of virulence gene promoters, like that for *bfpA*, under a variety of environmental conditions. In this way, factors that positively or negatively regulate virulence gene expression could be identified. Initial results from experiments investigating carbon source regulation of *bfpA* promoter

expression in the lux reporter strain appeared to confirm those of Vanmaele *et al.* (101). Wild type EPEC grown in the carbon source galactose displayed reduced levels of *bfpA* expression when compared to that in organisms grown in glucose. We therefore proposed that the wild type *bfpA* promoter is regulated by different carbon sources in the same way as the cloned *bfpA* promoter in the *lux* reporter strain. To test this theory, we grew wild type EPEC E2348/69 in the same carbon sources that the *lux* reporter strain was grown in, and monitored *bfpA* expression levels and LA to HEp-2 cells.

Materials and Methods

The following is a list of materials, reagents, and bacterial growth media used during the course of investigations.

Tryptic soy agar (TSA): 30 g/L (Difco-Becton Dickinson, Sparks, MD), 15 g/L agar (Gibco BRL-Life Technologies, Burlington, ON)

Luria-(broth) agar + kanamycin (LB^{km}) or ampicillin (LB^{Ap}): 10 g/L NaCl, 10 g/L tryptone-peptone, 5 g/L yeast extract, 15 g/L agar, 50 μg/ml kanamycin (Sigma, Oakville, ON) or 100 μg/ml ampicillin (Sigma)

Tryptic soy broth (TSB): 30 g/L (Difco)

Tryptic soy broth + kanamycin (TSB^{km}): 30 g/L (Difco), 50 μg/ml kanamycin (Sigma)

Luria-broth (LB): 10 g/L NaCl, 10 g/L tryptone-peptone, 5 g/L yeast extract

Dulbecco's modified Eagle medium (DMEM): catalogue no. 23800 (Gibco BRL), supplemented with 44 mM $NaHCO_3$, 40 μM phenol red, and 0.4% (w/v) carbon source;

one of D-glucose (Fisher Scientific), L-arabinose (Sigma), D-Gluconic acid sodium salt (Sigma), galactose (Fisher), D-glucosamine hydrochloride (Fisher)

Minimal essential medium (MEM): catalogue no. 41500 (Gibco), supplemented with 10% fetal bovine serum (FBS) (Sigma).

Tissue culture grade trypsin (0.25% v/v) in FC buffer (0.14 M NaCl, 5 mM KCl, 20 mM Tris-HCl, 5 mM Tris base, 0.5 mM EDTA [pH 7.2-7.4])

Phosphate buffered saline (PBS) without calcium and magnesium: 27 mM KCl, 15 mM KH_2PO_4, 1.36 mM NaCl, 81 mM Na_2HPO_4.

Giemsa stain:1:25 (v/v) giemsa (Fisher) in buffer : 0.07 M Na_2HPO_4 and 0.07 M $KH_2\ PO_4$

Anti-maltose binding protein (MBP) antibody (α-MBP): rabbit antiserum, New England BioLabs, Mississauga, ON

Rabbit anti-BfpA antibody (α-BfpA): generously provided by Dr. M.S. Donnenberg, University of Maryland, Baltimore, MD

Goat anti-rabbit IgG-Horse radish peroxidase conjugate (α-rabbit-IgG-HRP): Sigma

Enhanced chemiluminescence (ECL) western blotting detection reagents: Amersham Pharmacia Biotech, Baie d'Urfe, QC

Bovine serum albumin (BSA): Sigma

Tris-buffered saline (TBS): 50 mM Tris-HCl pH 7.5, 150 mM NaCl

Tris-buffered saline 0.1%Tween-20 (TTBS): 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) tween-20.

4X SDS-PAGE sample buffer: 0.5 M Tris pH 6.8 (1.2 ml), 10% w/v sodium dodecyl sulfate (SDS) (2 ml), glycerol (1 ml), 0.5% (w/v) bromophenol blue (0.5 ml), water to 9.5 ml, 50 mM dithiothreitol (DTT)

Bacterial strains

EPEC E2348/69 and JPN15 were kindly provided by Dr. R. P. Vanmaele (University of Alberta, Edmonton, AB). EPEC E2348/69 (O127:H6) is a wild type strain isolated from an infant with gastroenteritis (62). JPN15 is a derivative of E2348/69 spontaneously cured of the EAF plasmid associated with the LA phenotype (3, 63). Strains EPEC(pMS420) and EPEC(pLux/σ70) were constructed and provided by Dr. M. G. Surette (University of Calgary, Calgary, AB,). Plasmid pMS420 was constructed by cloning the *bfpA* promoter region from the EAF plasmid of E2348/69 into pCS26 upstream of a promoterless *luxCDABE* operon from *Photorhabdus luminescens* (Figure 3). The resulting plasmid pMS420 was isolated from *E. coli* DH5α, and transformed back into EPEC E2348/69 generating E2348/69(pMS420). The cloned *bfpA* promoter was confirmed by DNA sequencing. As a control a σ70 promoter region was cloned into pCS26, and transformed into EPEC E2348/69 generating E2348/69(pLux/σ70).

EPEC growth profiles in carbohydrate supplemented DMEM

EPEC E2348/69 and JPN15 TSB cultures (3 ml) were inoculated from a single colony and incubated 17-18h at 37°C. Isolated colonies of E2348/69(pMS420) and (pLux/σ70) were similarly inoculated into 3 ml TSB^{km} and incubated under the same conditions. At this time, sterile carbohydrate supplemented DMEM was pre-incubated at 37°C, 5% CO_2 overnight. 1:100 dilutions of overnight grown bacteria were subsequently cultured into the pre-equilibrated carbohydrate supplemented DMEM. Prior to inoculating E2348/69(pMS420) or (pLux/σ70) into carbohydrate supplemented DMEM, 50 μg/ml kanamycin was added to the media. Bacteria were incubated at 37°C, 5% CO_2 and the optical density (O.D.) at 600 nm measured every hour. Aliquots were also taken hourly to determine bacterial colony forming units (CFUs). Alternatively, overnight E2348/69(pMS420 or pLux/σ70) cultures were diluted 1:300 into DMEM (+ 25 μg/ml km) supplemented with the different carbon sources (personal communication with Dr. Surette). Bacteria were grown in the wells of a 96-well tissue culture plate (Costar 3631), incubated at 30°C, 5%CO_2 and placed in a Wallac Victor2 multiwell plate reader for measurements of light absorbance ($O.D._{620}$) and luminescence every hour.

Sample preparation for measuring $O.D._{600}$ and CFU/ml determination

Acid production by bacteria grown in tissue culture media such as DMEM changes the color of the media from red to yellow. To ensure that media color did not affect O.D. measurements, the bacteria were centrifuged at 14000 rpm for approximately three minutes. The supernatant was removed and the pellet re-suspended in PBS prior to measuring the $O.D._{600}$ using a Milton Roy Spectronic 21 photospectrometer. Sterile PBS

was used as a blank. To determine the number of CFU/ml for each culture, 100 µl aliquots were taken hourly and serially diluted to 10^7 in TSB. 100 µl of the 10^5, 10^6, and 10^7 dilutions were plated in triplicate on TSA for E2348/69 and JPN15, or LB^{km} for E2348/69(pMS420) and (pLux/σ70). Plates were incubated overnight at 37°C and colonies counted the following day.

EPEC growth prior to LA binding assay

Frozen glycerol stocks (-70 °C) of wild type EPEC E2348/69 and JPN15 were streaked onto TSA for isolated colonies and incubated overnight at 37°C. EPEC(pMS420) and EPEC(pLux/σ70) were streaked onto LB^{km} and incubated overnight under the same conditions. The following day, an isolated colony of EPEC E2348/69 and JPN15 as well as EPEC(pMS420) and EPEC(pLux/σ70) was inoculated into 3 ml TSB or TSB^{km} respectively, and incubated statically overnight (17-18h) at 37°C. A 1:100 dilution of each strain was sub-cultured into 3.5 ml carbohydrate-supplemented DMEM that had been pre-incubated overnight at 37°C, 5% CO_2. Cultures were incubated at 37°C, 5% CO_2 until mid-log phase growth was achieved (Table 2). Carbohydrate-supplemented DMEM for EPEC(pMS420) and EPEC(pLux/σ70), also contained 50 µg/ml kanamycin, which was added to the media prior to inoculation.

Preparation of semi-confluent HEp-2 cell monolayers

HEp-2 cells (CCL-23) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Confluent HEp-2 cell monolayers were grown in MEM supplemented with 10% FBS at 37°C in a humidified 5% CO_2 incubator. The monolayers were treated with 0.25% trypsin solution in FC buffer, and resuspended in fresh MEM + 10% FBS. Approximately 5 x 10^4 cells / 500 µl were added to the appropriate number of wells of a 24-well tissue culture plate (Falcon 3047) containing a sterile glass coverslip. The plate was incubated overnight at 37°C, 5% CO_2. The resulting semi-confluent HEp-2 cell monolayers were used the following day for the EPEC LA binding assay.

EPEC HEp-2 cell LA binding assay

EPEC strains E2348/69, JPN15, E2348/69(pMS420), and E2348/69(pLux/σ70) were grown until early to mid log phase in carbohydrate supplemented DMEM. Semi-confluent HEp-2 cell monolayers in 24-well tissue culture plates were washed once with sterile PBS prior to adding EPEC. In the presence of 1% (w/v) mannose approximately 1.9 x 10^8 bacterial cells were added. After 30 minutes incubating EPEC with HEp-2 cells at 37°C, 5% CO_2, coverslips were washed thoroughly five times with PBS to remove any unbound bacteria. Methanol was added for 10 minutes to fix adherent organisms, and the coverslips stained with giemsa for 20 minutes. Standard light microscopy (100x objective lens) was used to observe HEp-2 cells for LA. HEp-2 cells containing compact clusters of five or more EPEC were considered positive for the LA phenotype.

Table 2: EPEC growth rates in DMEM supplemented with various carbon sources

Strain/carbohydrate	Time (hrs) to reach early to mid-log phase
E2348/69 / glucose	3
galactose	26
arabinose	4
glucosamine	4
gluconate	4
JPN15 / glucose	3
E2348/69 (pMS420)/ glucose	3
galactose	26
E2348/69 (pLux/σ70)/glucose	3

Determination of *bfpA* expression

EPEC strains E2348/69, JPN15, E2348/69(pMS420), and E2348/69(pLux/σ70) were grown until early to mid log phase in carbohydrate supplemented DMEM as described previously. For each EPEC strain and carbohydrate source, approximately 6 x 10^7 bacterial cells were centrifuged at 14000 rpm for approximately 3 min. Once the supernatant was removed, the bacterial pellet was re-suspended in 20 µl PBS and 80 µl 4X SDS-PAGE sample buffer, and boiled for 10 min (whole cell lysates). Whole cell lysates were analyzed by 12.5% SDS-PAGE. Proteins were electrophoretically transferred to immobilon-P membranes (Millipore, Bedford, MA) and the membranes blocked overnight in 3% (w/v) BSA in TTBS at 4°C. The membranes were cut into two sections (divided above the 29.9 kDa MWS). As an internal control α-MBP (1:15000 in TTBS containing 0.05% (w/v) BSA) was incubated with the top membrane section, while the bottom was incubated with α-BfpA (1:7000) for 45 and 90 minutes respectively at room temperature. Membranes were washed with TTBS containing 0.05% (w/v) BSA and subsequently incubated with goat α-rabbit-IgG-HRP (1:15000 in TTBS containing 0.05% (w/v) BSA) for 60 minutes at room temperature. Membranes were washed with TBS, and ECL was performed to detect MBP and BfpA expression levels according to the manufacturer's instructions.

Results

Effect of carbon source on EPEC growth rates

Dr. Surette reported no measurable differences in the growth rates at 30°C of the EPEC reporter strains compared to wild type, or between strains carrying the *bfpA* reporter plasmid compared to the σ70 promoter control vector (data not shown, personal communication). Similarly, we did not observe measurable differences in the growth rates of wild type EPEC (which normally doubles every three hours), JPN15, or either of the EPEC reporter strains when the bacteria were grown at 37°C in DMEM supplemented with glucose, arabinose, glucosamine, or gluconate (Figures 4, 5). However when wild type E2348/69, or the reporter strain E2348/69(pMS420), were grown in the presence of galactose, growth rates decreased. Cultures required 26 hours to reach early to mid log phase growth compared to 3-4 hours for the other carbohydrates (Table 2).

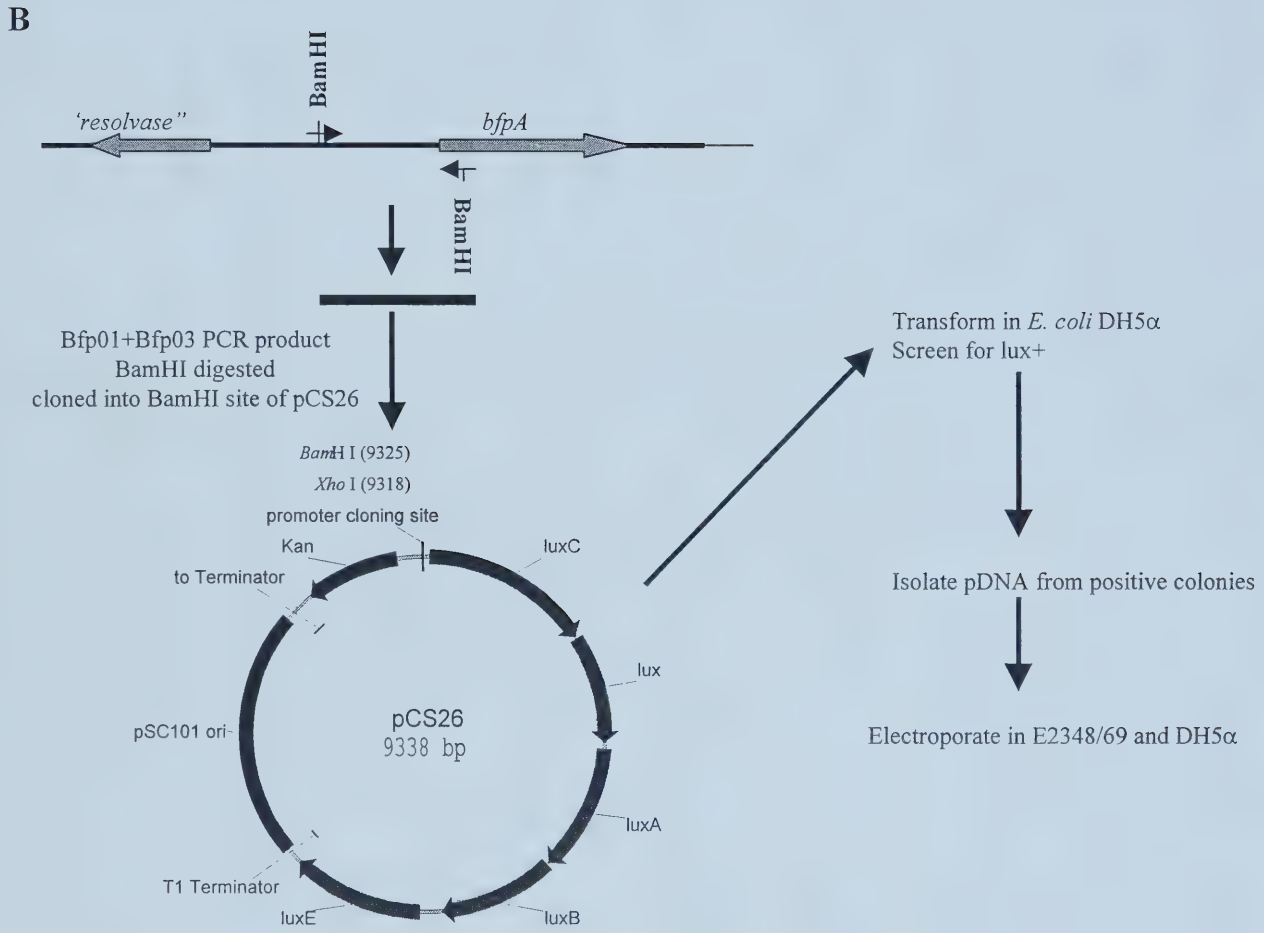
Figure 3: Construction of pMS240 (*lux* reporter plasmid)

A. Amplification of *bfpA* promoter region: Published EAF plasmid sequence from prototype EPEC strain B171 (Genbank Accession NC_002142) was used by Dr. Surette (University of Calgary) to design primers for PCR amplification of the *bfpA* promoter region including putative regulatory regions.

B. Making pMS420: Plasmid pCS26 is a low copy number vector containing the promoterless *lux*CDABE operon from *Photorhabdus luminescens*. Dr. Surette sub-cloned the PCR-amplified *bfpA* promoter region into the BamHI site of pCS26 just upstream of the promoterless *lux* operon. The resulting construct was then transformed into *E. coli* DH5α. Transformed colonies were selected by growing the bacteria in the presence of 25 μg/ml kanamycin. Plasmid DNA (now called pMS420) was isolated from DH5α lux+ clones and electroporated into EPEC E2348/69 to study *bfpA* promoter expression activity.

```
2101  AACATGTGGT GACGCTGGTC CGGCATATAT CAGAGGAGGG CGCTCACTTC
      TTGTACACCA CTGCGACCAG GCCGTATATA GTCTCCTCCC GCGAGTGAAG

   -2 euMetHisHis ArgGlnAsp ProMet
2151  CGGTGCTAGG AGGTAAGCCG GCGTGGCACC TGTTGAGGTG GCCATCCGCC
      GCCACGATCC TCCATTCGGC CGCACCGTGG ACAACTCCAC CGGTAGGCGG

2201  GGAGTGCCAC CGCGGTCGCA AAATCAACGC CCGGGGCGAT CGGCAATAAC
      CCTCACGGTG GCGCCAGCGT TTTAGTTGCG GGCCCCGCTA GCCGTTATTG

2251  GAAACGCGTT CTGGTGAATT CTGCAGGGGA ATAATGTTGT TCATCGGCTA
      CTTTGCGCAA GACCACTTAA GACGTCCCCT TATTACAACA AGTAGCCGAT

2301  TTTTTCACGT AGCGCACGCA CTGGTCATGG ATACAGTTAT TCTGGCCTGA
      AAAAAGTGCA TCGCGTGCGT GACCAGTACC TATGTCAATA AGACCGGACT

2351  AAGGCCCGAA CCTGCCATCA CAATAAATCG CAGCGCGCGG ATCCTTTTGT
      TTCCGGGCTT GGACGGTAGT GTTATTTAGC GTCGCGCGCC TAGGAAAACA
                                              Bfp01 →
2401  TGTCTTATCC CGCATTATTG CGGGAAAAAC CAAATGCGTA AAGGATCCTT
      ACAGAATAGG GCGTAATAAC GCCCTTTTTG GTTTACGCAT TTCCTAGGAA

2451  TTTCTGCTCA AATCCTCCAT GAAGCCAGTC ATGACGCGGG CTGAGGGCTG
      AAAGACGAGT TTAGGAGGTA CTTCGGTCAG TACTGCGCCC GACTCCCGAC

2501  GTTTACGGTT TTTGGGAGAA ATGGGGTAAT GCCAGGAGTA CCGGAAGTCA
      CAAATGCCAA AAACCCTCTT TACCCCATTA CGGTCCTCAT GGCCTTCAGT

2551  AATTCATGGG GATATTGACT GTTTATGTAC TGGGGGGGAC GGAAATATAT
      TTAAGTACCC CTATAACTGA CAAATACATG ACCCCCCCTG CCTTTATATA

2601  AAAAAAAAAA GAAAAAAAGA TTATTTTTTT TCTTGGTGCT TGCGTGTCTT
      TTTTTTTTTT CTTTTTTTCT AATAAAAAAA AGAACCACGA ACGCACAGAA

2651  TTTTAGTTTT AAGATTATTC CGTGACCTAT TAATACGGGG GTTTTATAAG
      AAAATCAAAA TTCTAATAAG GCACTGGATA ATTATGCCCC CAAAATATTC

   +2              MetValSer LysIleMet AsnLysLys TyrGluLysG
2701  GAAAACAGTT TTTATGGTTT CTAAAATCAT GAATAAGAAA TACGAAAAAG
      CTTTTGTCAA AAATACCAAA GATTTTAGTA CTTATTCTTT ATGCTTTTTC

   +2 lyLeuSerLeu IleGluSer AlaMetValLeu AlaLeuAla AlaThrVal
2751  GTCTGTCTTT GATTGAATCT GCAATGGTGC TTGCGCTTGC TGCCACCGTT
      CAGACAGAAA CTAACTTAGA CGTTACCACG AACGCGAACG ACGGTGGCAA
                CTAACTTAGA CGTTACCtaG gACGCGAACG
                     Bfp03   ←
```

A *bfp*A promoter region: Dr. Surette used two primers (bfp01 and bfp03) to generate a PCR product that has BamHI sites at each end. This includes the putative control regions for this promoter.


B
BamHI
'resolvase"
bfpA
BamHI
Bfp01+Bfp03 PCR product
BamHI digested
cloned into BamHI site of pCS26
BamHI (9325)
XhoI (9318)
promoter cloning site
Kan
luxC
to Terminator
lux
pSC101 ori
pCS26
9338 bp
luxA
T1 Terminator
luxE
luxB
Transform in E. coli DH5α
Screen for lux+
Isolate pDNA from positive colonies
Electroporate in E2348/69 and DH5α

BfpA promoter expression in pMS420

Expression from the cloned *bfpA* promoter in pMS420 is positively regulated in wild type E2348/69 background compared to *E. coli* DH5α (Figure 6). This finding demonstrates the usability of E2348/69(pMS420) for monitoring the cloned *bfpA* promoter activity. Presumably the *bfpA* promoter in pMS420 is under the control of the wild type EAF plasmid-encoded BfpT activator complex, as *E. coli* DH5α does not possess this regulator.

E2348/69(pMS420) grown in DMEM supplemented with different carbon sources at 30°C results in variable expression from the *bfpA* promoter (Figure 7). Growing the bacteria at 30°C slows the growth rate such that slight changes in *bfpA* promoter expression can be monitored. In response to the carbohydrates tested, *bfpA* promoter expression could be categorized into three groups. The carbon sources glucose, arabinose, and glucosamine appeared to elicit high levels of *bfpA* expression. E2348/69(pMS420) grown in DMEM with galactose or fructose, and glycerol or gluconate as carbon sources results in medium and low levels of *bfpA* expression respectively (Figure 7). This data suggests that carbohydrate source in the growth environment plays a role in the regulation of *bfpA* expression in EPEC(pMS420), and was hypothesized to similarly regulate *bfpA* expression in wild type E2348/69.

Figure 4: EPEC E2348/69 growth curve in DMEM supplemented with different carbohydrates

Overnight E2348/69 cultures (TSB) were sub-cultured (1:100) into pre-eqilibrtaed DMEM supplemented with either glucose, arabinose, glucosamine, or gluconate. Overnight JPN15 (plasmid cured derivative of wild type EPEC) was sub-cultured into DMEM supplemented with glucose. All DMEM cultures were grown at 37°C in a 5% CO_2 incubator. At hourly intervals and for each carbohydrate and EPEC strain, DMEM-grown bacteria were centrifuged and re-suspended in PBS prior to measuring optical density at 600 nm as described previously. Each growth curve was performed in triplicate such that each data point represents the average $O.D._{600}$. Error bars indicate the standard deviation.

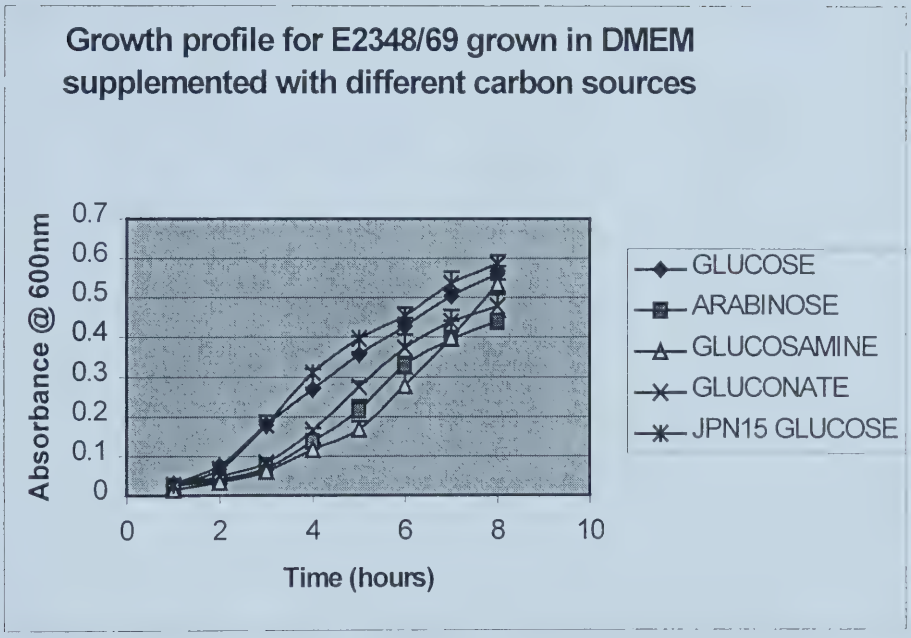
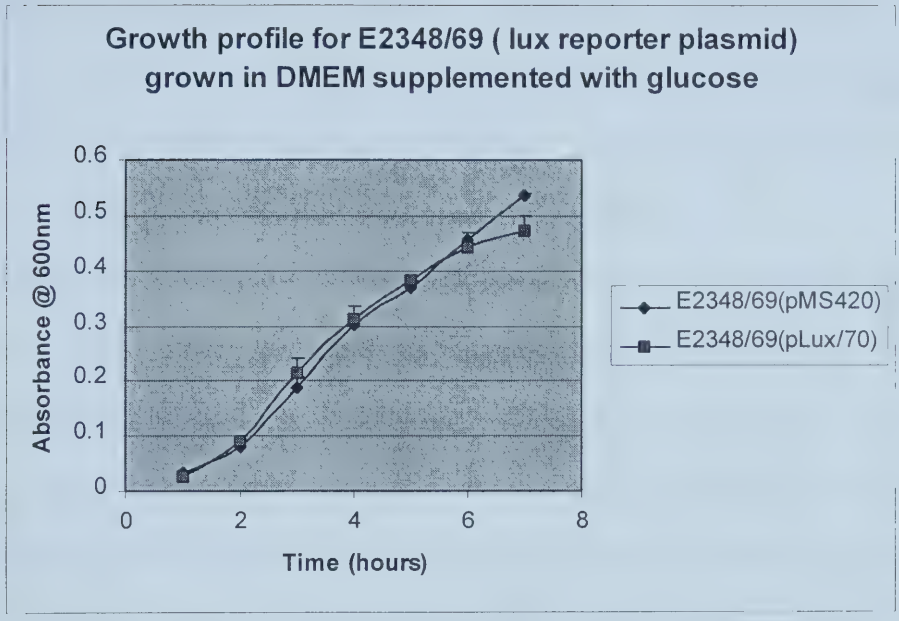

Growth profile for E2348/69 grown in DMEM supplemented with different carbon sources
Absorbance @ 600nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
Time (hours)
GLUCOSE
ARABINOSE
GLUCOSAMINE
GLUCONATE
JPN15 GLUCOSE

Figure 5: E2348/69 (lux reporter plasmid) growth curves in DMEM supplemented with glucose

EPEC *lux*-reporter strains E2348/69(pMS420) and (pLux/σ70) were grown overnight in LB containing 50 μg/ml kanamycin. The next morning cultures were inoculated (1:100) into pre-equilibrated DMEM containing glucose as the carbon source. Just prior to inoculation, 50 μg/ml kanamycin was added to the media. Cultures were grown at 37°C in a 5% CO_2 incubator. At hourly intervals and for each EPEC *lux*-reporter strain, DMEM-grown bacteria were centrifuged and re-suspended in PBS prior to measuring optical density at 600 nm as described previously. Each growth curve was performed three times such that each data point represents the average $O.D._{600}$. Error bars indicate the deviation about each data point.


Growth profile for E2348/69 (lux reporter plasmid) grown in DMEM supplemented with glucose
Absorbance @ 600nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
Time (hours)
E2348/69(pMS420)
E2348/69(pLux/70)

BfpA expression in wild type EPEC E2348/69

EPEC E2348/69 was grown in DMEM supplemented with glucose, galactose, arabinose, glucosamine, and gluconate until early to mid log phase growth was attained (Table 2). Additionally E2348/69(pMS420) was grown in DMEM supplemented with either glucose or galactose, while E2348/69(pLux/σ70) and JPN15 were grown in DMEM containing glucose (Table 2). In each case whole cell lysates of approximately 6 x 10^7 bacterial cells were analyzed by SDS-PAGE (Figure 8). Different carbon sources utilized by the EPEC strains did not appear to alter their protein expression profiles.

Proteins were transferred to Immobilon-P membranes, and immunoblots performed by incubation with either α-MBP or α-BfpA to detect MBP and BfpA expression respectively (Figure 9). BfpA is expressed when wild type EPEC utilizes glucose as a carbon source, but its expression decreases when galactose is used (Figure 9A, lanes 1 and 3 respectively). EPEC JPN15 (the plasmid cured derivative of wild type) does not express BfpA (Figure 9A, lane 2). There is little variation in BfpA expression levels when wild type EPEC is grown in DMEM supplemented with arabinose, glucosamine, or gluconate, relative to growth in glucose as a carbon source (Figure 9A, lanes 4, 5 and 6 relative to lane 1). However, the corresponding levels of MBP expression indicate that wild type EPEC grown in arabinose and gluconate as carbon sources appear to express increased levels of BfpA relative to those grown in glucose (Figure 9A, lanes 4 and 6 relative to lane 1). Conversely the coomassie-stained SDS-PAGE of the same samples indicates that approximately equal numbers of cells were loaded in each lane (Figure 8A, lanes 1, 4 and 6).

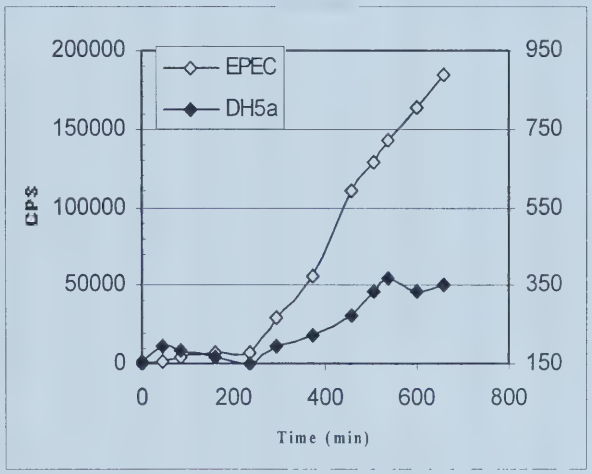
EPEC LA to HEp-2 cells

For EPEC strains grown in carbohydrate supplemented DMEM approximately 1.9 x 10^8 organisms were added to semi-confluent HEp-2 cell monolayers and incubated for 30 minutes at 37°C, 5%CO_2 to allow for bacterial attachment. The binding assay was performed in the presence of 1% mannose, which is required during EPEC attachment to HEp-2 cells to inhibit *E. coli* type I pili mediated binding (8). Wild type EPEC grown in glucose, arabinose, glucosamine, and gluconate all attached to HEp-2 cells with the same efficiency (approximately 80% LA). Wild type EPEC grown in galactose, JPN15 (plasmid cured derivative of wild type) grown in glucose, and strains with the cloned *bfpA* or σ70 promoter grown in glucose or galactose as the carbohydrate source did not attach to HEp-2 cells (Figure 10).

Figure 6: BFP promoter activity is up-regulated in EPEC E2348/69 background compared to *E. coli* DH5α

E. coli DH5α(pMS420) and E2348/69(pMS420) were grown overnight in LB containing 25 μg/ml kanamycin. The next day cultures were inoculated (1:300) into LB+25 μg/ml kanamycin in the wells of a 96-well tissue culture plate and incubated at 37°C. Note that LB was chosen because DH5α does not grow in DMEM. At hourly time points the plate was removed from the incubator and placed into a Wallac Victor 2 multi-well plate reader for measuring luminescence (counts per second). DH5α luminescence is scaled on the right verticle, and EPEC on the left verticle axis. There is an increase in E2348/69(pMS420) light output over time at levels well over DH5α background. These experiments were performed at the University of Calgary in Dr. Surette's laboratory.

Light Production *vs* time for DH5α/pMS420 and EPEC/pMS420 in LB



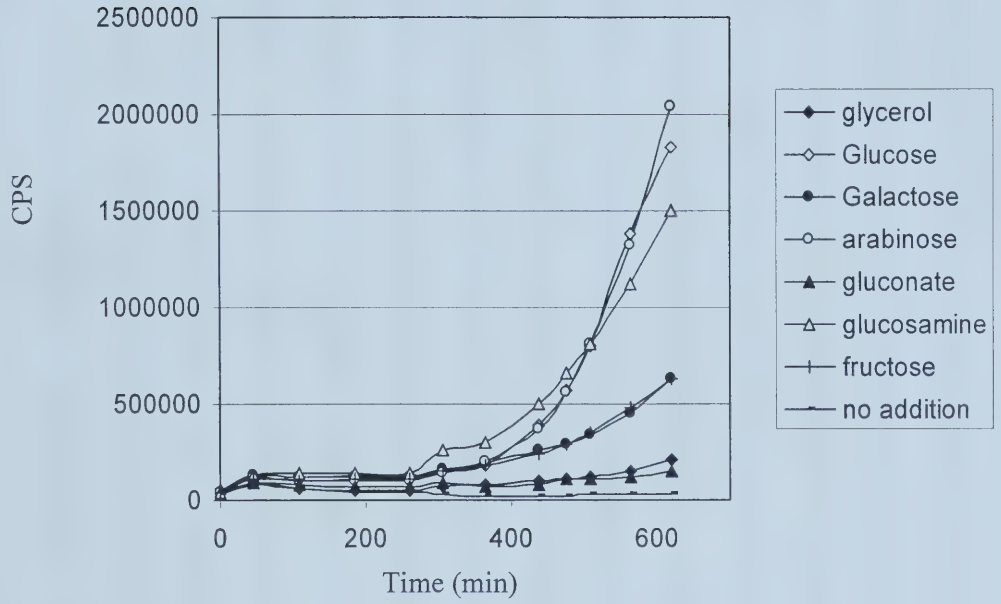
Discussion

We predicted that carbohydrates would regulate *bfpA* expression in wild type EPEC in a similar manner as *bfpA* promoter expression in the lux reporter strain E2348/69(pMS420). We grew wild type EPEC in DMEM supplemented with glucose, which is known to induce expression of BfpA and the LA phenotype (104). We also grew wild type EPEC in DMEM supplemented with arabinose or glucosamine, which are carbohydrates that induce a high level of *bfpA* promoter expression in the *lux* reporter strain E2348/69(pMS420) (Figure 7). Relative to *bfpA* expression when glucose is the carbon source, wild type EPEC that utilize arabinose or glucosamine had equivalent levels of *bfpA* expression (Figure 9A, lanes 4 and 5 relative to lane 1), consistent with *bfpA* promoter expression in the *lux* reporter strain. However, relative to glucose, MBP expression levels when arabinose and glucosamine are the carbon sources suggests that *bfpA* expression increases (Figure 9A, lanes 4 and 6). Yet the same SDS-polyacrylamide gel stained with coomassie indicates that approximately the same number of cells were loaded in each lane (Figure 8A, lanes 1, 4 and 6). We did not quantitate the intensity of the MBP bands in Figure 9A using densitometry as visually observing the immunoblot clearly revealed lower expression in lanes 4 and 6, when compared to lanes 1, 2, 3, and 5. MBP is a periplasmic protein encoded by the *malE* gene of the *mal* operon in *E. coli*. As there is little information available pertaining to carbohydrate regulation of the *mal* operon in *E. coli*, it seems plausible that MBP expression by *E. coli* grown in different carbon sources may not be an accurate internal control to ensure loading efficiency.

Figure 7: *bfpA* expression profile in E2348/69/pMS420 grown in DMEM with various carbohydrate sources

E2348/69(pMS420) was grown overnight in LB containing 25 μg/ml kanamycin. The following day an inoculum (1:300) of overnight bacteria was sub-cultured into carbohydrate-supplemented DMEM to which 25 μg/ml kanamycin was added. DMEM cultures were grown in 96-well tissue culture plates at 30°C in a 5% CO_2 incubator. Every hour the plate was removed from the incubator and placed into a Wallac Victor 2 multi-well plate reader for measuring luminescence (counts per second). Depending on the supplementary carbohydrate source present in DMEM, *bfpA* promoter expression could be categorized as low, medium, or high. These experiments were performed at the University of Calgary in Dr. Surette's laboratory.

bfpA Promoter Expression Profile



Expression at 30°C:	High	Medium	Low
	+ Glucose	+galactose	+glycerol
	+arabinose	+ fructose	+gluconate
	+glucosamine		

Wild type EPEC grown in DMEM containing galactose as opposed to glucose exhibited reduced *bfpA* expression, confirming previous results (101), and to some extent resembled the lack of *bfpA* expression in JPN15 (Figure 9A). Surprisingly, wild type EPEC grown in DMEM containing gluconate as a carbon source expressed *bfpA* to a similar degree as wild type EPEC grown in glucose (Figure 9A). This result was contrary to what was expected since E2348/69(pMS420) grown in gluconate-supplemented media produced less light than when it was grown in glucose. Contrary to *bfpA* promoter expression observed in the *lux* reporter strain (Figure 7), *bfpA* expression by wild type EPEC, grown in the presence of gluconate, exceeded the expression levels observed when the bacteria were grown in galactose (Figure 9A). This result indicates that *bfpA* promoter expression in the *lux* reporter strain does not precisely reflect *bfpA* expression in the wild type strain. Consistent with observations made by Dr. Surette, we observed a high rate of *bfpA* expression when the *lux* reporter strain E2348/69(pMS420) was grown in glucose (Figure 9B). When we grew E2348/69(pLux/σ70) in glucose-enriched DMEM *bfpA* expression disappeared completely (Figure 9B). While this strain does contain a *lux* reporter plasmid, it is in wild type E2348/69 background. It therefore possesses the wild type EAF plasmid, which encodes the *bfpA* gene as well as the positive regulator BfpT. Accordingly this strain was expected to express *bfpA* to the same degree as wild type EPEC. In some way, incorporation of the σ70/*lux*-reporter plasmid into wild type EPEC altered *bfpA* expression from the endogenous EAF plasmid.

The binding phenotype was found to correlate with *bfpA* expression levels as EPEC strains expressing *bfpA* attached to HEp-2 cells with the classical LA phenotype (Figure 10). One exception was the *lux* reporter strain E2348/69(pMS420), which expressed BfpA, but did

not adhere to HEp-2 cells with LA, or otherwise. While the BfpA subunit is expressed, the strain may be deficient in BFP assembly, which would account for the lack of binding activity observed. Transmission electron micrographs (TEM) of wild type E2348/69 and the *lux* reporter E2348/69(pMS420) both grown in glucose-enhanced DMEM revealed BFP in close association with wild type EPEC (Figure 11), but no BFP structures were observed in association with the *lux* reporter strain (Figure 12). While these results suggest that E2348/69(pMS420) does not assemble functional BFP on the cell surface, interpretation of this data is subjective, and the BFP observed in the wild type culture could be an artifact. In fact pilus-type structures were recently shown to be present in sterile culture media indistinguishable from pili observed in bacterial inoculated media (26). It is also possible that BfpA is not the primary adhesin responsible for initial EPEC attachment to tissue culture cells. BFP are not thought to be required for initial attachment in an *IVOC* model (37), leaving open the possibility that another adhesin involved in initial EPEC attachment awaits recognition. There is also speculation that EspA filaments rather than BFP are the mediators of initial non-intimate attachment to tissue culture cells by EPEC (54). Our finding that E2348/69(pMS420) which expresses BfpA, but does not bind to HEp-2 cells is consistent with these reports. Given our results, monitoring *bfpA* promoter activity by measuring light production in the *lux* reporter strain may not be an accurate way to predict *bfpA* expression or LA in the wild type strain. The *bfpA* promoter region has previously been cloned into a promoterless *cat* reporter vector and used to investigate factors that regulate wild type *bfpA* expression (78). The *lux* reporter system however may exhibit strain or species specificity and may not be compatible with EPEC E2348/69.

Measuring luminescence produced by *lux* reporter strains is an extremely sensitive method for detecting even small changes in promoter expression activity. Perhaps seemingly significant differences in promoter expression levels in the *lux* reporter strain are not reflected in monitoring protein expression levels in wild type EPEC using a more traditional approach. Further investigation is required before the EPEC *lux* reporter system can be used with confidence to identify factors that regulate wild type virulence gene expression.

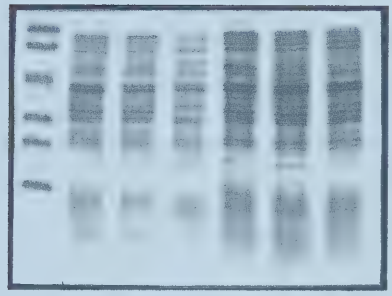
Figure 8: Protein analysis by SDS-PAGE for EPEC strains grown in various carbon sources

A. Whole cell lysates (WCL) for EPEC strains grown in DMEM supplemented with the carbon sources glucose, galactose, arabinose, glucosamine, and gluconate:

Overnight E2348/69 (TSB) cultures were sub-cultured (1:100) into pre-equilibrated DMEM containing the appropriate carbon source and incubated at 37°C in a 5% CO_2 incubator until early to mid-log phase growth was achieved (Table 2). Overnight JPN15 was similarly grown and sub-cultured into DMEM supplemented with glucose. For each EPEC strain and carbon source whole cell lysates from approximately 6 x 10^7 bacterial cells were analyzed by 12.5% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R250. Molecular weight standards (MWS) from top down: 112, 81, 49.9, 36.2, 29.2, and 21.3 kDa respectively. WCLs: Lane 1: E2348/69-glucose, Lane 2: JPN15-glucose, Lane 3: E2348/69-galactose, Lane 4: E2348/69-arabinose, Lane 5: E2348/69-glucosamine, Lane 6: E2348/69-gluconate.

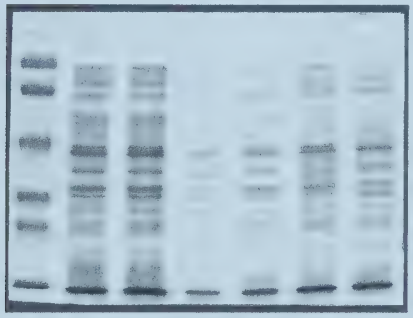
B. Whole cell lysates (WCL) for EPEC strains grown in DMEM supplemented with the carbon sources glucose or galactose:

Overnight E2348/69 (TSB) was sub-cultured (1:100) into pre-equilibrated DMEM containing either glucose or galactose as the carbon source. Overnight JPN15 was similarly grown and sub-cultured into DMEM supplemented with glucose. Overnight cultures (TSB^{km}) of E2348/69(pMS420) and (pLux/σ70) were sub-cultured (1:100) into DMEM supplemented with glucose or galactose containing 50 μg/ml kanamycin. All DMEM cultures were incubated at 37°C in a 5% CO_2 incubator until early to mid-log phase growth was achieved (Table 2). For each EPEC strain and carbon source whole cell lysates from approximately 6 x 10^7 bacterial cells were analyzed by 12.5% SDS-PAGE. The gel was stained with coomassie blue. Molecular weight standards (MWS) from top down: 112, 81, 49.9, 36.2, 29.2, and 21.3 kDa respectively. WCLs: Lane 1: E2348/69-glucose, Lane 2: JPN15-glucose, Lane 3: E2348/69-galactose, Lane 4: E2348/69(pMS420)-glucose, Lane 5: E2348/69(pLux/σ70)-glucose, Lane 6: E2348/69(pMS420)-galactose.



A

1 2 3 4 5 6



B

1 2 3 4 5 6

Figure 9: Detection of BfpA expression in EPEC strains grown in DMEM supplemented with various carbon sources

Detection of MBP and BfpA expression by immunoblot: EPEC strains E2348/69, JPN15, and E2348/69(pMS420 or pLux/σ70) were grown in DMEM supplemented with the carbon sources glucose, galactose, arabinose, glucosamine, and gluconate and WCLs analyzed by SDS-PAGE as described previously. Proteins were transferred to an Immobilon-P membrane and non-specific binding sites blocked with 3% (w/v) BSA. The membrane was cut into two sections for the purpose of incubating with two different primary antibodies. The top section, which contains the area between molecular weight standards 49.9 and 36.2 kDa, was incubated with α-MBP. The bottom section, which contains the area between molecular weight standards 29.2 and 21.3 kDa, was incubated with α-BfpA. Both sections were subsequently incubated with α-rabbit-IgG-HRP and the ECL system performed to visualize MBP and BfpA expression.

A. Lane 1: E2348/69-glucose, Lane 2: JPN15-glucose, Lane 3: E2348/69-galactose, Lane 4: E2348/69-arabinose, Lane 5: E2348/69-glucosamine, Lane 6: E2348/69-gluconate.

B. Lane 1: JPN15-glucose, Lane 2: E2348/69-glucose, Lane 3: E2348/69-galactose, Lane 4: E2348/69(pMS420)-glucose, Lane 5: E2348/69(pLux/σ70)-glucose

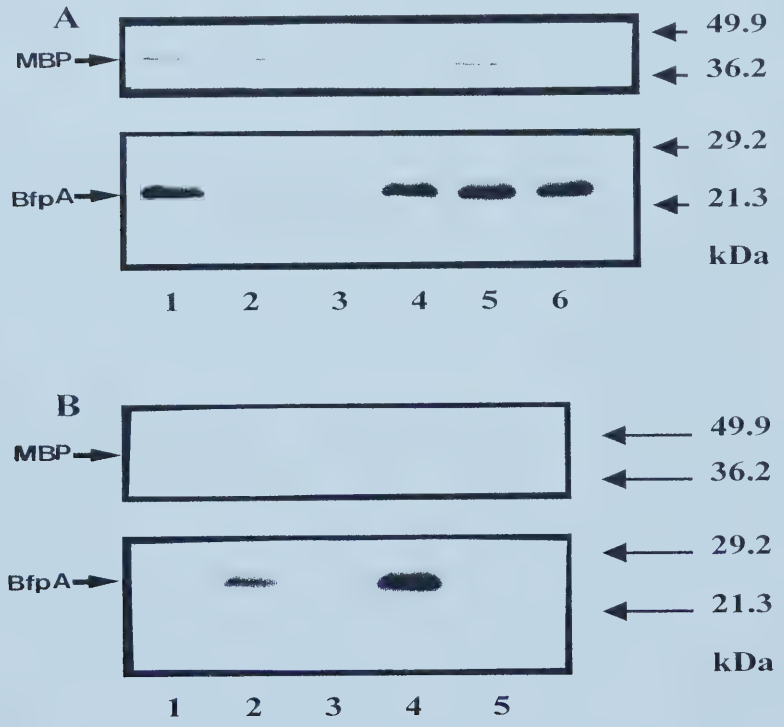

A
MBP
BfpA
49.9
36.2
29.2
21.3
kDa
1 2 3 4 5 6
B
MBP
BfpA
49.9
36.2
29.2
21.3
kDa
1 2 3 4 5

Figure 10: EPEC LA to HEp-2 cells subsequent to growth in various carbohydrates

EPEC strains were grown until mid-log phase in DMEM supplemented with various carbohydrate sources (Table 2) as previously described. Approximately 1.9 x 10^8 bacterial cells were added to semi-confluent HEp-2 cells on glass coverslips in 24-well tissue culture plates. Bacteria and HEp-2 cells were incubated for 30 minutes at 37°C in a 5% CO_2 incubator to allow for bacterial binding. Any bacteria that did not attach were washed away and the coverslips were treated with methanol for 10 min to fix attached bacteria. The coverslips were giemsa-stained and observed microscopically for the LA phenotype. Bars represent the average number of HEp-2 cells with localized adherent EPEC over three experiments. Error bars indicate the standard deviation.

GLC, GAL, ARA, INE, and ATE: LA of E2348/69 utilizing glucose, galactose, arabinose, glucosamine, or gluconate for carbon sources

JPN15: LA of JPN15 utilizing glucose as a carbon source

pMS420/GLC or GAL: LA of E234869 (pMS420) utilizing glucose or, galactose

pLux/70/GLC: LA of E234869 (pLux/σ70) utilizing glucose

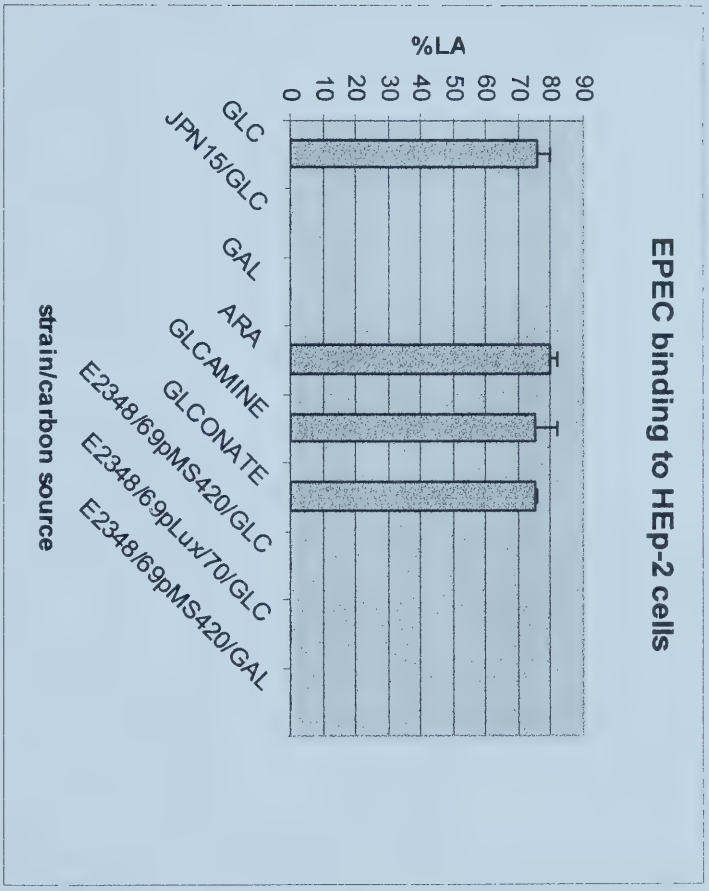

EPEC binding to HEp-2 cells
%LA
0
10
20
30
40
50
60
70
80
90
GLC
JPN15/GLC
GAL
ARA
GLCAMINE
GLCONATE
E2348/69pMS420/GLC
E2348/69pLux/70/GLC
E2348/69pMS420/GAL
strain/carbon source

Figure 11: Bundle-forming pili (BFP) in close association with wild type EPEC E2348/69

An E2348/69 overnight culture (TSB) was sub-cultured into DMEM supplemented with glucose and grown until early log phase (3 h) at 37°C in a 5% CO_2 incubator. The culture was then gently mixed, and a small aliquot placed on a standard formvar-coated transmission electron microscopy (TEM) grid. The sample was negatively stained using 1% PTA for TEM analysis.

BFP (rope-like) structures are observed in close association with E2348/69.

Bar = 100 nm

TEM performed by Richard Sherburne, University of Alberta, Edmonton, AB.

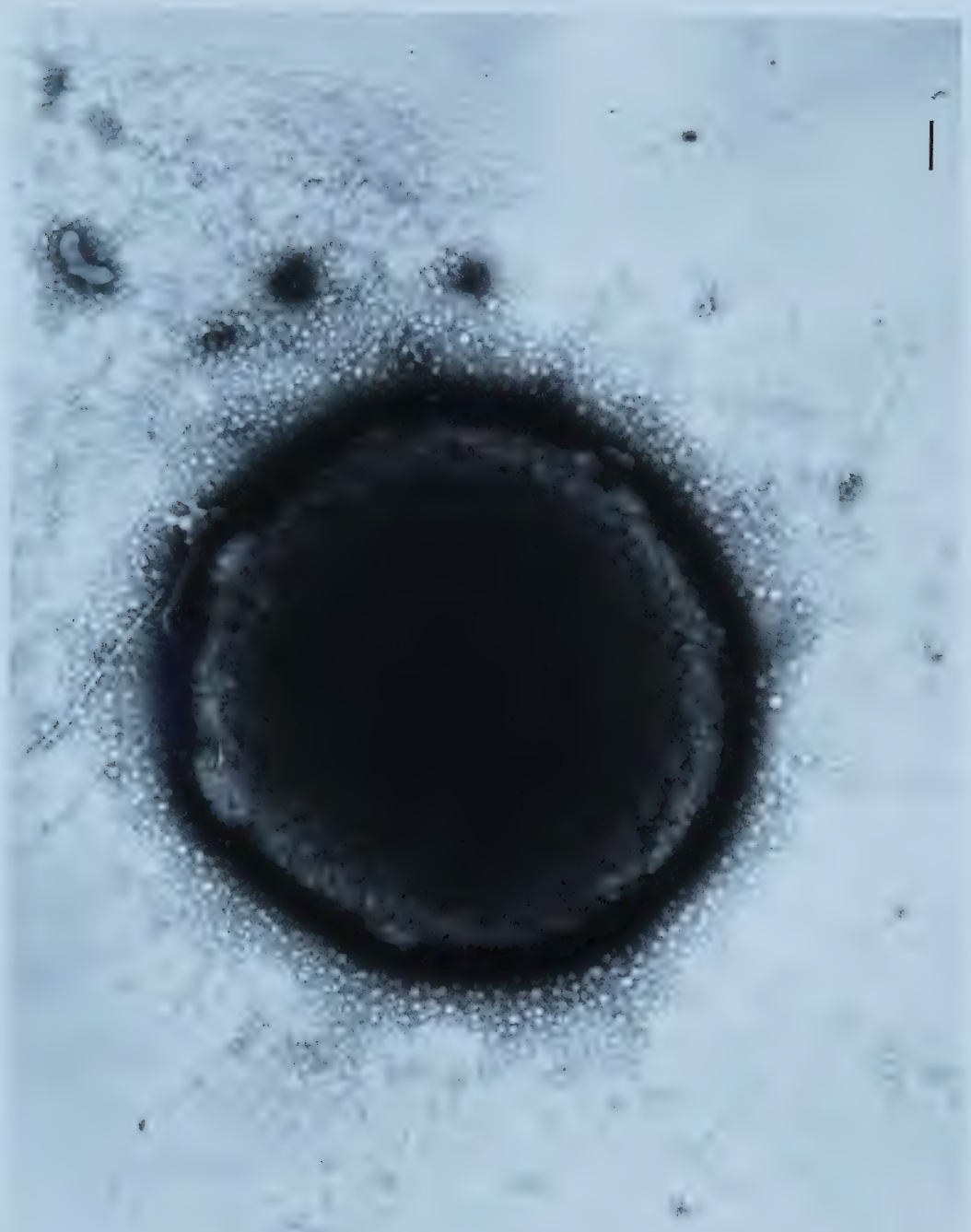
Figure 12: Bundle-forming pili (BFP) are not associated with the *lux* reporter strain E2348/69(pMS420)

An E2348/69(pMS420) overnight culture (LB^{km}) was sub-cultured into DMEM supplemented with glucose + 50 μg/ml and grown until early log phase (3 h) at 37°C in a 5% CO_2 incubator. The culture was then gently mixed, and a small aliquot placed on a standard formvar-coated transmission electron microscopy (TEM) grid. The sample was negatively stained using 1% PTA for TEM analysis.

BFP (rope-like) structures are not observed in association with E2348/69(pMS420).

Bar = 100 nm

TEM performed by Richard Sherburne, University of Alberta, Edmonton, AB.



Chapter 3

Investigating the cell-binding domain of Intimin (Int280)

Intimin's cell-binding domain resides within the C-terminal 280 amino acids (Int280). This portion of the protein facilitates Intimin binding to its receptor Tir on host cell surfaces (21, 35, 47). Originally thought to be of host cell origin, Tir is now known to be an EPEC protein, which is translocated into the host cell membrane where it serves as the Intimin receptor (48). An interaction between purified Int280 and HEp-2 cells that have not had Tir inserted in their membrane (i.e. have not been infected with EPEC), suggests that Intimin can also bind to host cells by a Tir-independent process (24). Reports of A/E lesions forming in the absence of phosphorylated Tir further support the belief that in addition to Tir, Intimin also recognizes a second receptor (10, 79). The fold of Int280 reveals a C-type lectin domain (45), which has been shown to bind to β1-integrins (20). In contrast, more recent evidence indicates that β1-integrins are not required for Intimin-mediated cell attachment or A/E lesions (64). Intimin expression decreases following A/E lesion formation (52), and following EPEC incubation with a synthetic carbohydrate conjugate (102). These results together with evidence that Int280 can bind to host cells in the absence of Tir, led us to hypothesize that Int280 contains a carbohydrate-recognition domain capable of binding to a eukaryotic cell surface receptor such as LeX. We designed a solid phase binding assay to determine whether Int280 possesses a functional carbohydrate-recognition domain and to gain further knowledge into the binding properties of Int280.

Materials and Methods

The following materials, reagents, and bacterial growth media (not identified in Chapter 2) were used in performing the experiments:

Lysis buffer: 50 mM Na_2HPO_4, 500 mM NaCl, 10 mM imidazole (Sigma)

p-Nitrophenyl phosphate (pNpp): 5 mg/ml pNpp disodium tablets (Sigma)

Diethanolamine: Fisher Scientific

Ni-NTA agarose protein purification system: Qiagen Inc. Mississauga, ON

BCIP/NBT: Western blot color development reagents (Sigma)

Isopropyl β-D-thiogalacto-pyranoside (IPTG): Sigma

BSA-glycoconjugates LeX-BSA, LeY-BSA, N-acetyl lactosamine-BSA (LacNAc-BSA): Alberta Research Council, Edmonton, AB

1% gelatin: 1% gelatin (Boehringer Mannheim, Laval, QC) solution (w/v) in PBS

Goat anti-rabbit IgG-alkaline phosphatase conjugate (α-rabbit-IgG-AP): Sigma

Goat anti-mouse IgG- alkaline phosphatase conjugate (α-mouse-IgG-AP): Sigma

Rabbit anti-intimin antibody (α-intimin): rabbit polysera generously provided by Dr. G. Frankel, University of London, London, UK

Mouse anti-histidine$_{(6)}$ antibody (α-his$_6$): monoclonal anti-polyhistidine (Sigma)

Mouse anti-bovine serum albumin antibody (α-BSA): monoclonal anti-BSA (Sigma)

Mouse anti-LeX antibody (α-LeX): ID Labs Inc. London, ON

Mouse anti-LeY antibody (α-LeY): ID Labs Inc. London, ON

Agarose: Gibco BRL

RNase: 500 μg/ml stock Boehringer Mannheim

EDTA: BDH Inc. Toronto, ON (0.5 M stock solution pH 8.0)

Skim milk powder: Carnation (Nestle, Don Mills, ON)

1% PTA: sodium phosphotungstic acid (PTA) (TAAB Laboratories, England)1% (w/v) in water

Construction of polyhistidine-Int280 (6xHis-Int280)

The C-terminal 939 bp of the E2348/69 *eae* gene was amplified by PCR. We designed primers (Gibco BRL, Burlington, ON) based on the published *eae* gene sequence (Genbank Accession AF022236) as follows:

Forward primer (INT2805'NHE1): CAGTGCTAGCATTACTGAGATTAAGGCTGA,

Reverse primer (INT2803'KPN1): TACGGGTACCTTATTTTACACAAGTGGCAT, introducing restriction sites Nhe1, and Kpn1 respectively (underlined). The resulting PCR product was analyzed on 1% agarose and could be visualized as a band of ca. 900 bp in size (data not shown). The band was purified using the QIAEX II Agarose Gel Extraction

kit (Qiagen Inc., Mississauga, ON) according to the manufacturer's instructions. Cloning vector pRSETB was isolated from *E. coli* DH5α (Invitrogen, Carlsbad, CA) using standard molecular biology techniques (85). pRSETB was then treated with RNase (0.25 μg/ml in water) for 30 minutes at 37°C followed by cleaving with the restriction enzymes Nhe1 and Kpn1 (Gibco BRL) for 2 hours at 37°C. pRSETB was gel-purified as described above. Int280 was cloned into cleaved pRSETB between the Nhe1 and Kpn1 sites and just downstream of the polyhistidine tag (Figure 13). The resulting construct was then transformed into *E. coli* DH5α. Transformants were selected by growing the bacteria in the presence of 100 μg/ml ampicillin. Using the above primers, PCR confirmed the presence of Int280 in 15 randomly chosen transformants. Plasmid DNA (pRSETB/Int280), was isolated and electroporated into the *E. coli* expression strain BL21(DE3) (Invitrogen, Carlsbad, CA). Positive clones were selected by growing the bacteria in the presence of 100 μg/ml ampicillin (for overall cloning strategy, see Figure 13). DNA sequencing confirmed the correct Int280 sequence.

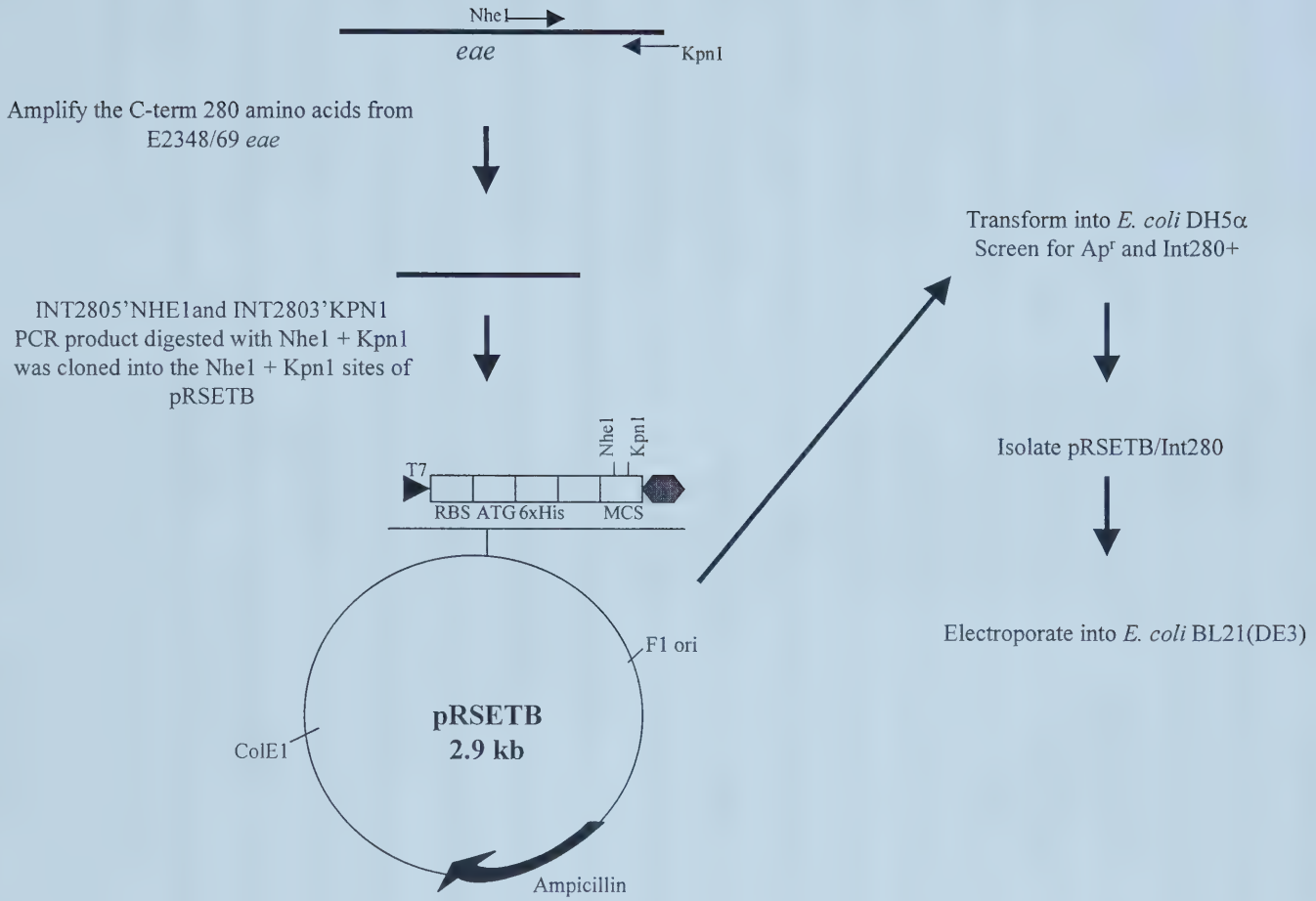
Expression and purification of 6xHis-Int280

Foreign protein expression in pRSETB is under the control of the T7 phage promoter. Transcription is initiated when BL21(DE3)-encoded RNA polymerase binds to the T7 promoter region, and is inducible by adding IPTG. BL21(DE3)pRSETB/Int280 or BL21(DE3)/pRSETB (5 ml culture from a single colony) were grown overnight in LB + 100 μg/ml ampicillin at 37°C, with shaking at approximately 200 rpm. Whole cell lysates of overnight cultures were analyzed by SDS-PAGE to ensure that only in BL21(DE3)pRSETB/Int280, but not in the empty vector control strain,

BL21(DE3)/pRSETB, did 6xHis-Int280 expression occur (Figure 14). Meanwhile 100 ml of overnight-grown BL21(DE3)pRSETB/Int280 was centrifuged (Beckman Avanti-J20 centrifuge) for 10 minutes at 4°C and 8000 rpm. The supernatant was discarded and the pellet washed once with cold PBS and resuspended in 2 ml lysis buffer. 1 μg/μl (w/v) lysozyme (Sigma) was added, and incubated on ice for approximately 30 minutes to lyse the cells. 6xHis-Int280 was purified using the Ni-NTA agarose protein purification system as per the manufacturer's instructions. Aliquots (10 μl) from each step of the purification procedure were mixed with 5 μl 4X SDS-PAGE sample buffer, boiled for 10 minutes and analyzed by SDS-PAGE to monitor 6xHis-Int280 purification (Figure 15).

Figure 13: Construction of pRSETB/Int280

Based on the published E2348/69 *eae* gene sequence (Genbank accession AF022236) we designed primers INT2805'NHE1, and INT2803'KPN1 to amplify the C-terminal 939 bp. Int280 was cloned into pRSETB between the Nhe1 and Kpn1 sites just downstream of the polyhistidine tag. The resulting construct was transformed into DH5α and the bacteria were grown in the presence of 100 μg/ml ampicillin. 15 randomly chosen colonies were screened for the presence of Int280 by PCR using the same primers. Plasmid pRSETB/Int280 was isolated from one such positive clone, and electroporated into *E. coli* BL21(DE3) for expressing 6xHis-Int280.


Nhe1
eae
Kpn1
Amplify the C-term 280 amino acids from E2348/69 eae
INT2805'NHE1and INT2803'KPN1 PCR product digested with Nhe1 + Kpn1 was cloned into the Nhe1 + Kpn1 sites of pRSETB
Nhe1
Kpn1
T7
RBS ATG 6xHis
MCS
F1 ori
pRSETB
2.9 kb
ColE1
Ampicillin
Transform into E. coli DH5α
Screen for Apr and Int280+
Isolate pRSETB/Int280
Electroporate into E. coli BL21(DE3)

Immunoblots of putative and purified 6xHis-Int280

Overnight cultures (LB + 100 μg/ml ampicillin) of BL21(DE3)pRSETB (empty vector control strain), and BL21(DE3)pRSETB/Int280 were sub-cultured (1:20) into fresh media and grown shaking at 200 rpm, 37°C until $O.D_{600}$ = 0.6. For each culture, as well as for an overnight grown wild type EPEC E2348/69 culture (TSB), aliquots were removed for preparing whole cell lysates for SDS-PAGE. For the BL21(DE3)pRSETB and BL21(DE3)pRSETB/Int280 cultures, we added IPTG [1 mM] for inducing 6xHis-Int280 expression, and contined to grow the cultures at 37°C, 200 rpm. After 4 hours whole cell lysates were prepared for each culture for SDS-PAGE. BL21(DE3)pRSETB/Int280 was grown for an additional 13 hours (17 hours in total), after which a final aliquot was taken for preparing a whole cell lysate. All cell lysates were separated by SDS-PAGE. Proteins were then electrophoretically transferred to Immobilon-P membranes and non-specific binding sites were blocked overnight with 3% (w/v) BSA in TTBS at 4°C. The membranes were subsequently incubated with either α-intimin, or α-his_6 antibodies diluted 1:3000 in TTBS + 0.05% BSA (w/v) at room temperature for 2 hours. After washing the membranes with TTBS + 0.05% BSA, they were incubated with secondary antibodies (1:10000 dilutions of α-rabbit-IgG-AP or α-mouse-IgG-AP) for α-intimin and α-his_6 respectively for 1 h at room temperature. The membranes were washed with TTBS + 0.05% BSA and specific protein interactions were visualized using the BCIP/NBT color development system (Figure 16).

Figure 14: 6xHis-Int280 is expressed in BL21(DE3)pRSETB/Int280

BL21(DE3)pRSETB/Int280 and BL21(DE3)/pRSETB were grown overnight in LB + 100 μg/ml ampicillin at 37°C. Whole cell lysates were analyzed by 12.5% SDS-PAGE and the gel stained with commassie blue. 6xHis-Int280 is expressed in BL21(DE3)pRSETB/Int280 (lane 2), but not in the empty vector control strain BL21(DE3)/pRSETB (lane 1).

35.6 kDa
6xHis-Int280
1
2

Figure 15: Purification of 6xHis-Int280

To ensure that 6xHis-Int280 was expressed only in BL21(DE3)pRSETB/Int280 and not BL21(DE3)pRSETB, whole cell lysates were prepared from overnight cultures. Overnight-grown BL21(DE3)pRSETB/Int280 was centrifuged to pellet the cells, which were then lysed using 1 μg/ml lysozyme in lysis buffer. 6xHis-Int280 was purified from the cell lysates using the Ni-NTA agarose protein purification system according to the manufacturer's instructions. Samples (10 μl) were collected from all steps of the process to monitor 6xHis-Int280 purification. Each sample was boiled with 5 μl 4X SDS sample buffer and analyzed by 12.5% SDS-PAGE. The gel was stained with coomassie blue.

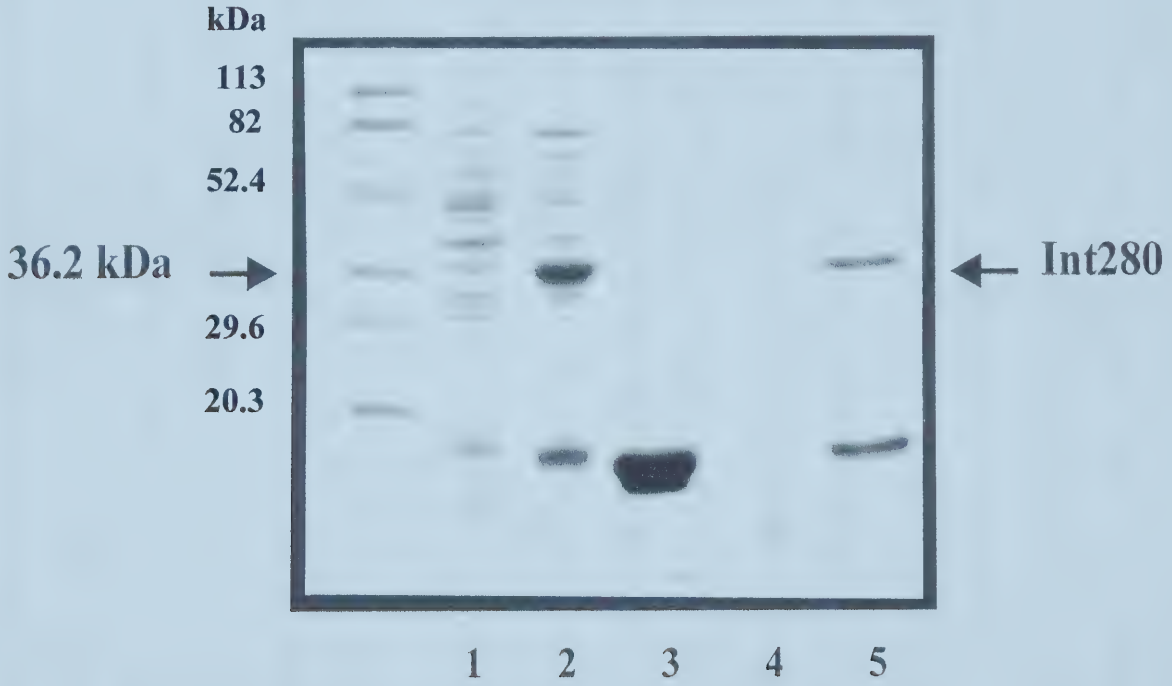
Lane 1: BL21(DE3)pRSETB whole cell lysate

Lane 2: BL21(DE3)pRSETB/Int280 whole cell lysate

Lane 3: Flow through (FT): proteins in the BL21(DE3)pRSETB/Int280 lysate that do not bind to the Ni-NTA agarose beads

Lane 4: Wash 4: any non-specific proteins that may have attached to Ni-NTA agarose beads removed after washing 4X with a wash buffer

Lane 5: 6xHis-Int280 eluted from Ni-NTA agarose beads using an elution buffer with a high concentration of imidazole


kDa
113
82
52.4
36.2 kDa
29.6
20.3
Int280
1
2
3
4
5

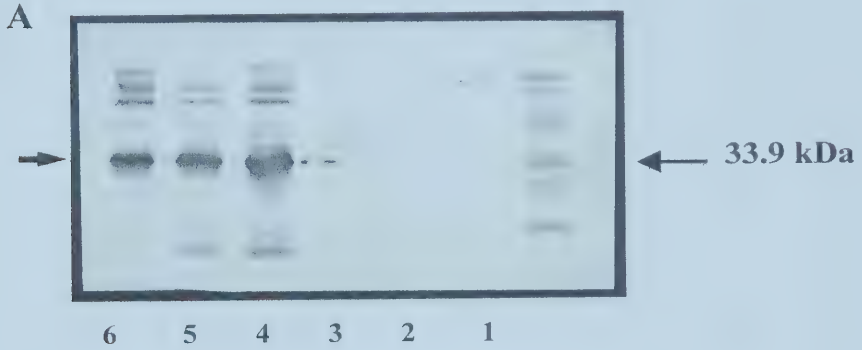
Two aliquots (10 µl) of purified 6xHis-Int280 that was eluted from the Ni-NTA agarose were mixed with 5 µl of 4X SDS-sample buffer, boiled for 10 minutes, and analyzed by SDS-PAGE. Proteins were electrophoretically transferred to an Immobilon-P membrane and blocked overnight with 3% (w/v) BSA in TTBS at 4°C. The membrane was washed with TTBS + 0.05% BSA and cut into two pieces vertically, between the duplicate samples, for the purpose of incubating with either α-intimin, or α-his_6 antibodies. Immunoblots were then carried out exactly as described above for putative 6xHis-Int280 (Figure 17).

Int280 solid phase binding assay (ELISA assay)

The wells of a 96-well EIA/RIA plate (Costar 3590) were coated with 5 µg/ml 6xHis-Int280 overnight at 4°C. Depending on the experiment, the plate was washed 4X with PBS, PBS + $CaCl_2$ and $MgSO_4$ (0.1 mM and 1 mM respectively), or PBS + 10 mM EDTA. Non-specific binding sites on the plastic were blocked with 100 µl of 1% gelatin for 1 h at 37°C. The plates were again washed 4X with PBS. Increasing concentrations (0-10 µg/ml) of LeX-BSA, or 10 µg/ml BSA alone (100 µl volumes) were then added to the 6xHis-Int280 bound to the plastic, and incubated for 1 h at 37°C. The plate was again washed 4X with PBS. To detect a specific binding interaction between 6xHis-Int280 and LeX-BSA or BSA, α-BSA (100 µl, diluted 1:8000 in PBS) was added to the wells and the plate incubated for 1 h at 37°C. As a control to ensure that the plastic was efficiently coated with 6xHis-Int280, adding BSA or LeX-BSA was omitted, and instead, α-intimin (1:4000 in PBS) was added for 1 h at 37°C.

Figure 16: α-intimin and α-his_6 antibodies specifically recognize putative 6xHis-Int280

Immunoblots of putative 6xHis-Int280 with α-intimin (A) and α-his_6 (B):
BL21(DE3)pRSETB/Int280 and BL21(DE3)pRSETB were grown overnight in LB containing ampicillin at 37°C and 200 rpm. Both strains were sub-cultured (1:20) into fresh media and grown until $O.D_{600}$ = 0.6. For each strain, an aliquot was then removed and whole cell lysates prepared. Both cultures were then induced by adding IPTG and continued to grow at 37°C and 200 rpm. Whole cell lysates were prepared after 4 h and 17 h (BL21(DE3)pRSETB/Int280 culture only). All whole cell lysates were analyzed by 12.5 % SDS-PAGE, and proteins were transferred to Immobilon-P membranes. 3% (w/v) BSA was used for blocking non-specific binding sites on the membranes. They were then incubated with α-intimin or α-his_6 and subsequently incubated with α-rabbit-IgG-AP or α-mouse-IgG-AP respectively. The BCIP/NBT color development system was performed for visualizing protein interactions. An approximately 34 kDa protein reacts with both α-intimin (A) and α-his_6 (B).
The protein samples (lanes 1-6) and molecular weight standards (MWS) are the same for both (A) and (B).
MWS (top to bottom): 111, 73, 47.5, 33.9, 28.8, 20.5 kDa
Lane 1: overnight-grown wild type EPEC E2348/69 whole cell lysate
Lane 2: BL21(DE3)pRSETB (empty vector control) whole cell lysate pre-IPTG
Lane 3: BL21(DE3)pRSETB (empty vector control) whole cell lysate 4 h post-IPTG
Lane 4: BL21(DE3)pRSETB/Int280 whole cell lysate pre-IPTG
Lane 5: BL21(DE3)pRSETB/Int280 whole cell lysate 4 h post-IPTG
Lane 6: BL21(DE3)pRSETB/Int280 whole cell lysate 17 h post-IPTG


A
33.9 kDa
6 5 4 3 2 1

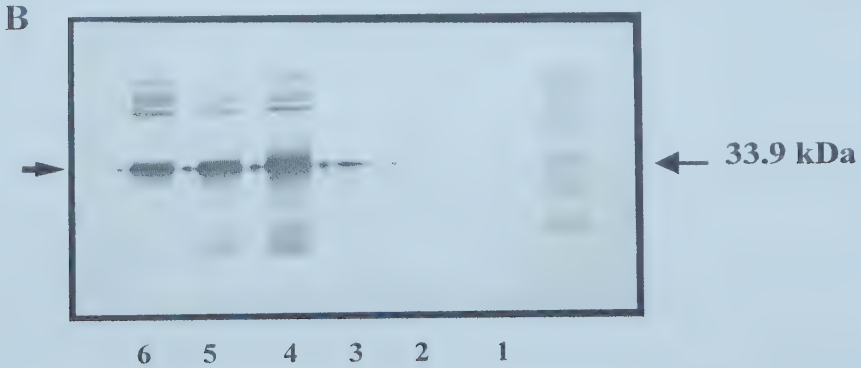
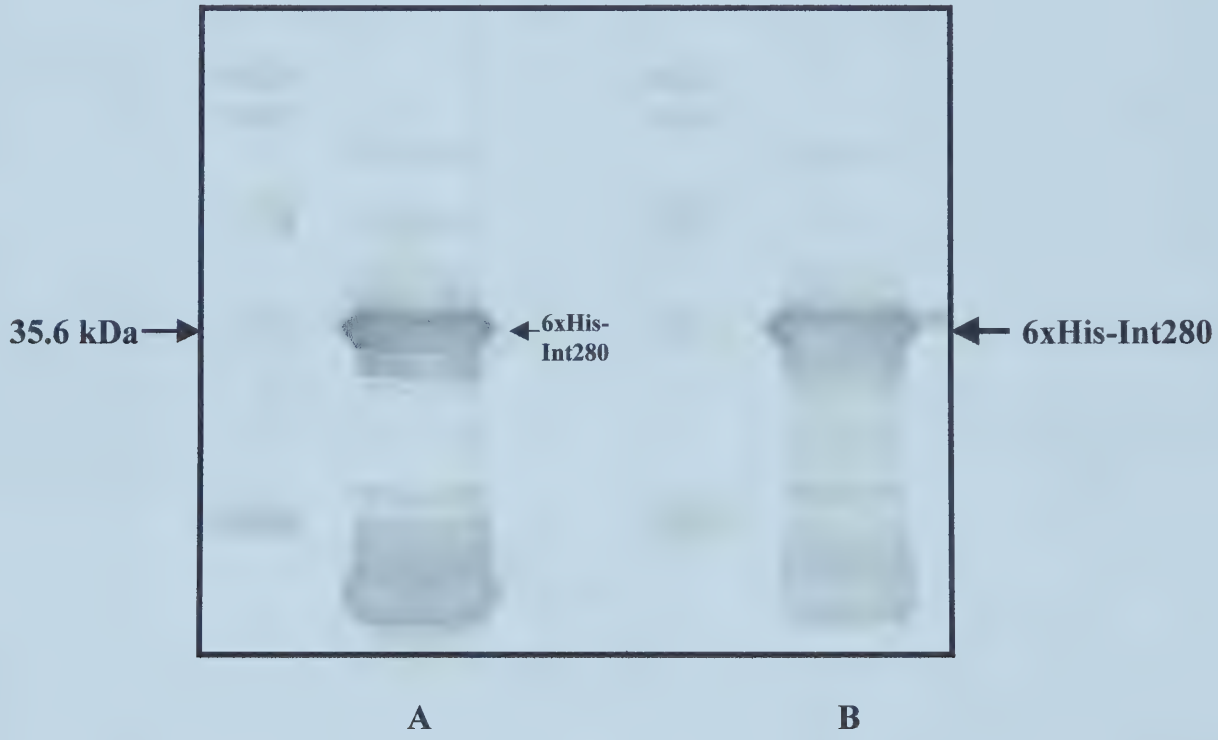

B
33.9 kDa
6 5 4 3 2 1

Figure 17: Purified 6xHis-Int280 reacts with α-intimin and α-his_6 antibodies

Immunoblot of purified 6xHis-Int280 with α-intimin (A) and α-his_6 (B):
Two aliquots of 6xHis-Int280 (10 μl) that were purified from Ni-NTA agarose as previously described were mixed with 5 μl 4X SDS sample buffer, boiled, and analyzed by 12.5% SDS-PAGE. Purified 6xHis-Int280 migrates to approximately the same position as the 35.6 kDa molecular weight marker. The proteins were then transferred to an Immobilon-P membrane, and 3% (w/v) BSA was used for blocking the non-specific binding sites. The membrane was cut vertically into two sections for incubating with either α-intimin (A), or α-his_6 (B). The membranes were next incubated with α-rabbit-IgG-AP (A), or α-mouse-IgG-AP (B). We used the BCIP/NBT color development system to detect protein interactions.


35.6 kDa
6xHis-
Int280
6xHis-Int280
A
B

To ensure that LeX was efficiently conjugated to BSA, a control was set up in which the plate was coated overnight with 5 μg/ml LeX-BSA, and following the washing and blocking steps described above, α-LeX (1:100 in PBS) was added for 1 h at 37°C. After incubating with the primary antibody, plates were washed 4X with PBS and the appropriate secondary antibody (alkaline phosphatase-conjugates diluted 1:2000 in PBS) were added for 1 h at 37°C (Table 3). Plates were washed once more, 4X, with PBS. Color-development substrate (200 μl), 1 mg/ml pNPP in [10% (w/v) diethanolamine in 0.5 mM $MgCl_2$], was added and the plate incubated for 30 minutes at 37°C. Light absorbance at 405 nm was measured using a Spectra Max 340 multi-well plate reader.

An alternative ELISA assay was developed to determine whether 6xHis-Int280 could specifically recognize BSA-glycoconjugates other than LeX-BSA. Wells (96-well EIA/RIA plates) were coated with 5 μg/ml BSA, LeX-BSA, LeY-BSA, or LacNAc-BSA overnight at 4°C. Plates were washed 4X with PBS containing 10 mM EDTA and blocked with 1% gelatin. Increasing concentrations (0-10 μg/ml) of 6xHis-Int280 were added (100μl) and the plates incubated for 1 h at 37°C. The plates were washed 4X and 100 μl primary antibody, α-his_6 (1:8000 in PBS) was added and the plates incubated for 1 h at 37°C. After washing again, 100 μl secondary antibody, α-mouse-IgG-AP (1:2000 in PBS) was added for 1 h at 37°C. Plates were washed once more, substrate added, and color development measured as described above.

Table 3:

Primary (1^o) and secondary (2^o) antibody combinations used in the Elisa assays

Protein(s) on plate	**1^o Ab**	**2^oAb**
(control wells)		
6xHis-Int280	α-intimin	α-rabbit-IgG-AP
	or α-his_6	α-mouse-IgG-AP
BSA	α-BSA	α-mouse-IgG-AP
LeX-BSA	α-BSA	α-mouse-IgG-AP
	or α-LeX	α-mouse-IgG-AP
LeY-BSA	α-LeY	α-mouse-IgG-AP
LacNAc-BSA	α-BSA	α-mouse-IgG-AP
(experimental wells*)		
6xHis-Int280::LeX-BSA	α-BSA	α-mouse-IgG-AP
6xHis-Int280::BSA	α-BSA	α-mouse-IgG-AP
BSA::6xHis-Int280	α-his_6	α-mouse-IgG-AP
LeX-BSA::6xHis-Int280	α-his_6	α-mouse-IgG-AP
LeY-BSA::6xHis-Int280	α-his_6	α-mouse-IgG-AP
LacNAc-BSA::6xHis-Int280	α-his_6	α-mouse-IgG-AP

*Experimental wells are those in which BSA-glycoconjugates and 6xHis-Int280 are added sequentially for the purpose of detecting a binding interaction. The protein listed before the double semi-colon (::) was coated on the plate overnight, and the protein listed after was subsequently added.

Immunoblots for detecting an interaction between 6xHis-Int280 and BSA-glycoconjugates

5 µl 4X SDS sample buffer was added to each of 15 µg BSA, LeX-BSA, LeY-BSA, LacNAc-BSA, or anti-his_6, boiled for 10 min and analyzed by SDS-PAGE. Proteins (glycoconjugates) were electrophoretically transferred to an Immobilon-P membrane, and the non-specific binding sites blocked overnight with 5% skim milk powder (w/v) in TTBS. The membrane was then incubated with 5 µg/ml 6xHis-Int280 in TTBS for 1 h at room temperature. After washing with TTBS, α-intimin (1:2000 in TTBS) was incubated with the membrane for 1 h at room temperature. The membrane was washed and incubated with α-rabbit-IgG-HRP (1:15000 in TTBS) for 1 h at room temperature. After washing a final time with TBS, ECL was performed according to the manufacturer's instructions to visualize specific 6xHis-Int280/BSA-glycoconjugate interactions.

Results

E. coli BL21(DE3) pRSETB/Int280 expresses a protein that specifically reacts with both α-his_6 and α-intimin antibodies

E. coli BL21(DE3) containing pRSETB with the cloned C-terminal 280 amino acids of EPEC E2348/69 Intimin, expresses a protein with a molecular weight of approximately 34 kDa (Figure 14). As Int280 has previously been reported to be approximately 30 kDa (21) this protein was thought to be the cloned 6xHis-Int280. Furthermore, the 34 kDa protein was not expressed in the empty vector control strain, BL21(DE3)pRSETB (Figure 14), or

in wild type EPEC (Figure 16A, lane 1), which does however express native Intimin (94 kDa), that is specifically recognized by anti-intimin. Further investigation revealed that the protein expressed by BL21(DE3)pRSETB/Int280 reacts with both α-intimin and α-his_6 antibodies (Figure 16, lanes 4-6) strongly suggesting that the protein was 6xHis-Int280. Interestingly, inducing BL21(DE3)pRSETB/Int280 with IPTG did not enhance 6xHis-Int280 expression, suggesting that leaky expression from the T7 promoter was likely sufficient. 6xHis-Int280 was purified (Figure 15), and as expected, specifically reacted with both α-intimin and α-his_6 antibodies (Figure 17) indicating that we had in fact purified the correct protein. The additional lower molecular weight bands seen in Figure 17 most likely represent incomplete 6xHis-Int280 translation products. This explanation is supported by the fact that these bands still react with the intimin and his_6 antibodies, and must retain sufficient portions of the intimin epitope, and the his-tag in order to do so. Furthermore, using protease inhibitors while purifying 6xHis-Int280 could not prevent the appearance of these lower molecular weight bands (data not shown).

6xHis-Int280 does not specifically bind to LeX-BSA or other BSA-glycoconjugates in an ELISA assay

Contrary to our hypothesis, we failed to observe a specific binding interaction between LeX-BSA and 6xHis-Int280 using the solid phase binding assay. Regardless of whether the assays were carried out in the presence of PBS, PBS + $CaCl_2$ and $MgSO_4$, or PBS + 10 mM EDTA we could not detect 6xHis-Int280 binding to LeX-BSA above background levels using BSA alone (Figure 18). In all three cases, positive controls indicated that 6xHis-Int280 was sufficiently coated on the plastic, and should binding occur between

LeX-BSA and 6xHis-Int280, it would be detectable by using α-BSA. These results revealed that while the ELISA assay was clearly functioning, we were still unable to show 6xHis-Int280 binding to LeX-BSA. Detecting LeX-BSA with α-LeX confirmed that the carbohydrate was properly conjugated to BSA (Figure 19). We also tried switching the ELISA sandwich order such that various BSA-glycoconjugates were coated on the plate, and increasing concentrations of 6xHis-Int280 were added. However, we still did not observe 6xHis-Int280 binding to LeX-BSA, or any of the other BSA-glycoconjugates tested above BSA background levels (Figure 20). This assay was working accurately as positive controls indicated that BSA and the various BSA-glycoconjugates were sufficiently coated on the plastic, and that the carbohydrates were definitely conjugated to BSA. Using α-his_6, any binding between 6xHis-Int280 and BSA-glycoconjugates would have been detected.

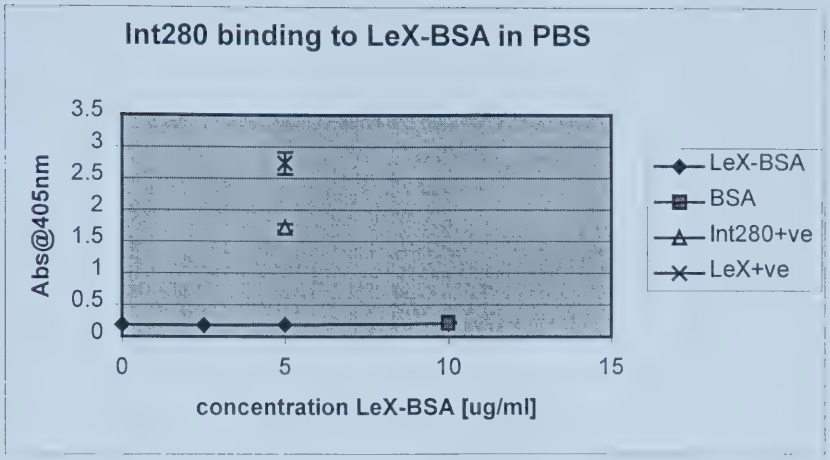
6xHis-Int280 does not specifically bind to LeX-BSA or other BSA-glycoconjugates by immunoblotting

When 6xHis-Int280 was incubated with an Immobilon-P membrane containing BSA, LeX-BSA, LeY-BSA, or LacNAc-BSA no specific binding interaction was detected. However, 6xHis-Int280 did positively recognize α-his_6 on the membrane (data not shown).

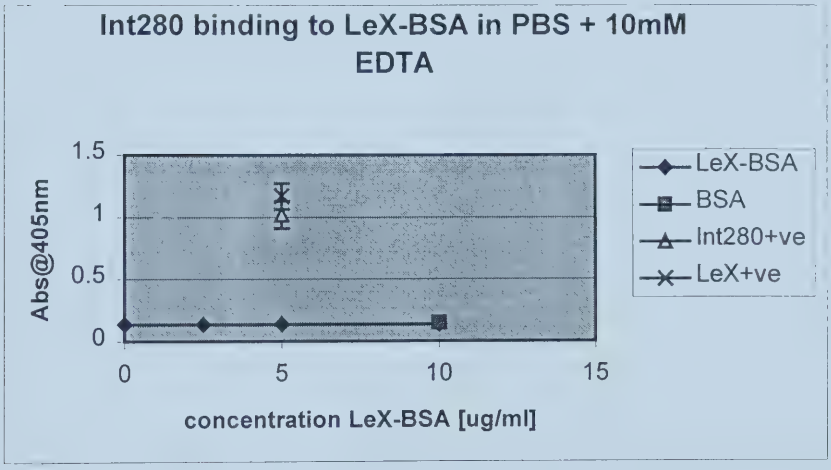
Figure 18: 6xHis-Int280 does not bind to LeX-BSA in an ELISA assay

96-well plates were coated with 5 μg/ml 6xHis-Int280. The plate was washed 4X with PBS (**A**), PBS + 10 mM EDTA (**B**), or PBS + $CaCl_2$ and $MgSO_4$ (**C**). Non-specific binding sites on the plastic were blocked with 1% gelatin. After washing with PBS, increasing concentrations (0-10 μg/ml) of LeX-BSA, or 10 μg/ml BSA alone were then added to the 6xHis-Int280 bound to the plastic, and incubated for 1 h at 37°C. The plates were washed again with PBS. To detect a specific binding interaction between 6xHis-Int280 and LeX-BSA or BSA, α-BSA (1:8000 in PBS) was added to the wells and the plate incubated for 1 h at 37°C. For the Int280+ve control wells the intermediate step of adding BSA or LeX-BSA was omitted. Instead α-intimin (1:4000 in PBS) was added for 1 h at 37°C. The LeX+ve control wells were coated with LeX-BSA overnight. Following the washing and blocking steps described above, α-BSA (1:8000 in PBS) was added for 1 h at 37°C. After incubating with the primary antibodies, plates were washed with PBS and secondary antibodies α-rabbit-IgG-AP and α-mouse-IgG-AP for detecting α-intimin and α-BSA respectively were added for 1 h at 37°C (see Table 3 for antibody combinations). Substrate [pNPP in (10% (w/v) diethanolamine in 0.5 mM $MgCl_2$)] was incubated with the plates for 30 minutes and color-development monitored by measuring light absorbance at 405 nm. Each data point represents the average value over three experiments with error bars indicating the standard deviation.

A


Int280 binding to LeX-BSA in PBS
Abs@405nm
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
concentration LeX-BSA [ug/ml]
LeX-BSA
BSA
Int280+ve
LeX+ve

B


Int280 binding to LeX-BSA in PBS + 10mM EDTA
Abs@405nm
1.5
1
0.5
0
0
5
10
15
concentration LeX-BSA [ug/ml]
LeX-BSA
BSA
Int280+ve
LeX+ve

C

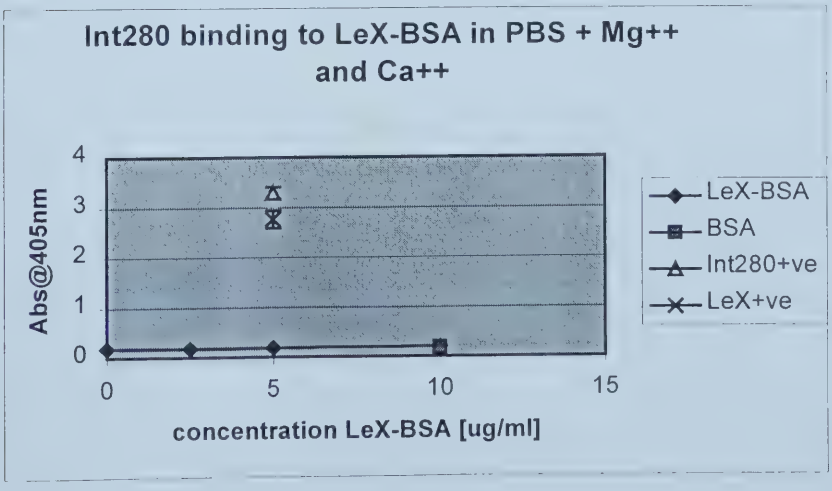

Int280 binding to LeX-BSA in PBS + Mg++ and Ca++
Abs@405nm
4
3
2
1
0
0
5
10
15
concentration LeX-BSA [ug/ml]
LeX-BSA
BSA
Int280+ve
LeX+ve

Figure 19: 6xHis-Int280 does not bind to LeX-BSA in an ELISA assay

6xHis-Int280 was used to coat a 96-well plate. The plate was washed with PBS + 10 mM EDTA and non-specific binding sites on the plastic were blocked with 1% gelatin. After washing again, increasing concentrations (0-10 μg/ml) of LeX-BSA, or 10 μg/ml BSA alone were added to the 6xHis-Int280 bound to the plastic, and incubated for 1 h at 37°C. The plates were washed again with PBS. To detect a specific binding interaction between 6xHis-Int280 and LeX-BSA or BSA, α-BSA (1:8000 in PBS) was added to the wells and the plate incubated for 1 h at 37°C. The Int280+ve control was performed exactly as described for Figure 18. The LeX+ve control was performed exactly as described for Figure 18 except that α-LeX (1:100 in PBS) was the primary antibody used for ensuring that LeX was properly conjugated to BSA. After incubating with primary antibody and washing the plate with PBS, α-mouse-IgG-AP was added for 1 h. Color development was measured exactly as described in Figure 18. Each data point represents the average value over three experiments with error bars indicating the standard deviation.

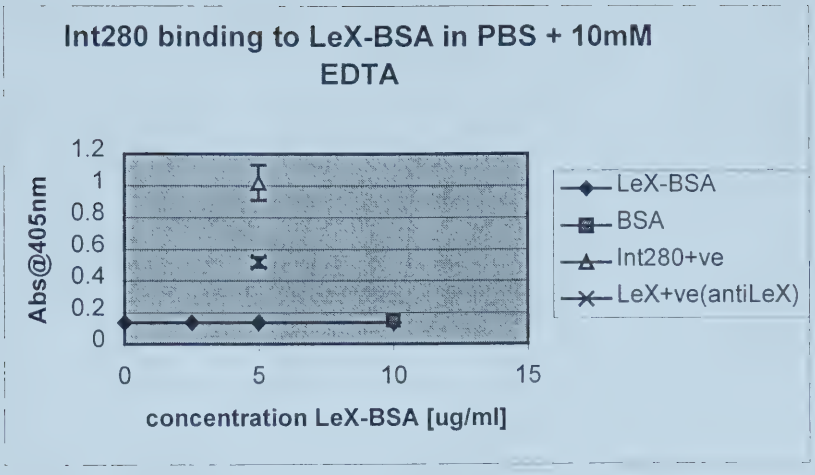
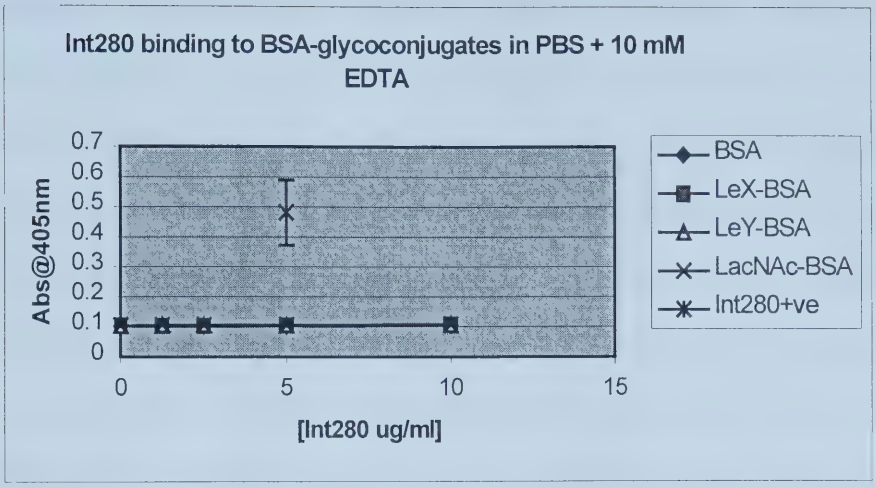

Int280 binding to LeX-BSA in PBS + 10mM EDTA
Abs@405nm
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
concentration LeX-BSA [ug/ml]
LeX-BSA
BSA
Int280+ve
LeX+ve(antiLeX)

Figure 20: 6xHis-Int280 does not bind to BSA-glycoconjugates in the ELISA assay

Wells were coated with 5 μg/ml BSA, LeX-BSA, LeY-BSA, or LacNAc-BSA overnight. Plates were washed with PBS containing 10 mM EDTA and blocked with 1% gelatin. Increasing concentrations (0-10 μg/ml) of 6xHis-Int280 was added and the plates incubated for 1 h at 37°C. After washing again, primary antibody, α-his_6 (1:8000 in PBS) was added and the plates incubated for 1 h at 37°C. Adding secondary antibody, α-mouse-IgG-AP (1:2000 in PBS), substrate, and measuring color production was performed exactly as described for Figure 18. Monitoring sugar conjugation to BSA was achieved by coating wells with LeX, LeY, or LacNAc-BSA, followed by incubating with primary antibodies α-LeX (1:100), α-LeY (1:100), and α-BSA (1:8000) respectively, as α-LacNAc was not readily available (see Table 3 for antibody combinations). The Int280+ve control was performed exactly as per Figure 18, except that α-his_6 (1:8000) was the primary antibody. Each data point represents the average value over three experiments with error bars indicating the standard deviation.


Int280 binding to BSA-glycoconjugates in PBS + 10 mM EDTA
Abs@405nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
[Int280 ug/ml]
BSA
LeX-BSA
LeY-BSA
LacNAc-BSA
Int280+ve

Discussion

Intimin is known to bind to Tir on host epithelial cell surfaces, which facilitates intimate EPEC binding. Tir is an EPEC-encoded protein that is translocated by way of the Type III secretion system into the host cell plasma membrane where it serves as the Intimin receptor (48). Previous studies have demonstrated that the cell-binding activity of Intimin is located within the C-terminal 280 amino acids (Int280), and that purified Intimin is capable of binding to mammalian cells in the absence of Tir (21, 24). The fold (45) and crystal structure (66) have recently revealed that Int280 contains a domain resembling C-type lectins. This information suggests a role for protein-carbohydrate interactions in intimin-mediated attachment and gives rise to the possibility that in addition to Tir, Intimin may recognize a second receptor of eukaryotic origin. A reduction in Intimin expression has been observed following A/E lesion formation (52) and following pre-incubating EPEC with a synthetic glycoconjugate (102). Taken together, these results led us to hypothesize that Int280 might be capable of binding to a host cell surface receptor such as LeX. There is no evidence suggesting that calcium or metal ion binding is required for Int280 activity. Accordingly, we first investigated binding activity between 6xHis-Int280 and the synthetic glycoconjugate LeX-BSA by performing a solid phase binding assay using PBS that did not contain any calcium, or magnesium (Figure 18 A). This approach failed to demonstrate Int280 binding activity. We speculated that perhaps traces of calcium ions were present in the water used to make the PBS and that this could in fact be inhibiting a binding interaction. We therefore tried adding 10 mM EDTA to the PBS and performed the assay again (Figure 18 B) but were unsuccessful at demonstrating that Int280 recognizes LeX-BSA. Performing the assay using PBS containing calcium and magnesium similarly did

not permit 6xHis-Int280 binding to LeX-BSA. Despite these results, we continued to test whether Int280 might specifically recognize LeX-BSA or other BSA-glycoconjugates. Using a slightly different approach that involved coating ELISA plates with different BSA-glycoconjugates and then adding 6xHis-Int280 in the presence of EDTA, we were still unable to observe Int280 binding activity (Figure 20). Immunoblotting BSA-glycoconjugates with 6xHis-Int280 was equally ineffective at demonstrating a binding interaction (data not shown).

Though we could not demonstrate 6xHis-Int280 binding to BSA-glycoconjugates by means of the various ELISA assays or immunobloting, this does not exclude the possibility that Int280 possesses a functional carbohydrate recognition domain. Perhaps our procedures were not sensitive enough to detect low affinity carbohydrate-protein interactions. Despite the multivalent nature of the BSA-glycoconjugates used in the ELISA assays, oligomerization of the 6xHis-Int280 ligand would be necessary in order to increase the binding affinity. The presence of the N-terminal his-tag may have altered Int280 folding, thereby inhibiting its binding to the carbohydrates tested. Yet another possibility is that the presence of 6xHis-Int280 degradation products could also have been blocking a binding interaction between 6xHis-Int280 and carbohydrate. Isolating the approximately 35 kDa 6xHis-Int280 (Figure 15) from any possible degradation products, and using only that product in the ELISA assay could resolve this matter. The observed decrease in intimin expression when EPEC were pre-incubated with Le^X-BSA was very slight compared to expression levels when the bacteria were incubated with BSA alone (102). It is therefore possible that Int280 specifically recognizes a cell surface carbohydrate yet to be identified, and that Le^X and the BSA-glycoconjugates we tested do not function as

Intimin receptors. Integrins have been proposed to serve as intimin receptors as Int280 has been shown to bind to β1-integrins (20). A contradictory report suggests that β1-integrins are not required for intimin-mediated attachment, or in forming A/E lesions (64). Furthermore, intimin lacks the proper charge grouping necessary for binding to integrin (66), which makes integrin an unlikely candidate for a second intimin receptor. Still another possibility is that Int280 does not possess a functional carbohydrate recognition domain considering the lack of calcium binding residues typically associated with C-type lectins (45, 66). Further investigating the binding activity of the Int280 C-type lectin-like domain is necessary for discovering the existence of a host derived carbohydrate (or other) Intimin receptor.

General Discussion

Non-intimate attachment to epithelial cells in the small intestine is thought to be the first step in the EPEC infection process. However, unambiguous identification of the adhesin responsible for this initial binding has not occured. Current theories propose that bundle-forming pili (BFP), long projections from the bacterial surface (EspA filaments), or some yet to be identified adhesin may facilitate initial adherence (Figure 1). BFP are a member of the type IV family of pili (12, 87), which are produced by many pathogenic bacteria and thought to promote bacterial adherence to host cells (4). For the human pathogens, *Neisseria gonorrhoeae*, *Vibrio cholerae* and *Pseudomonas aeruginosa* type IV pili have been shown to be required for infectivity and virulence *in vivo* (17, 36, 93). In the case of EPEC infection, evidence suggesting that BFP are involved in host cell adherence comes mainly from *in vitro* observations. By growing EPEC in the tissue culture media, DMEM, *bfpA* expression is induced (104). Under such conditions, expression of BFP corresponds to the autoaggregation phenotype and does not require epithelial cells, or their products (104). Such aggregates may facilitate EPEC adherence to tissue culture cells in a distinct pattern termed localized adherence (LA) (4, 8, 86, 104). The absence of *bfpA* expression and BFP assembly results in the disappearance of the LA phenotype *in vitro*. Indeed EPEC may exhibit BFP-mediated LA to host cells *in vivo* as small bowel biopsies of EPEC infected children reveal discrete microcolonies of bacteria attached to mucus membranes (79, 83, 84). Further evidence suggesting that BFP are required for virulence *in vivo* was noted when, subsequent to having endured the natural course of EPEC infection, children produced IgG antibodies to BfpA that could specifically recognize purified BFP from wild

type EPEC (67). As the production of antibodies against bacterial antigens is a good indication that those antigens are produced *in vivo*, this report suggests a role for BFP in natural infections. Interestingly, adding anti-BFP antibodies to tissue culture cells together with wild type EPEC resulted in reduced LA compared to when only bacteria were added (28). Adult human volunteers who ingested EPEC strains incapable of producing BfpA were observed to experience reduced volumes of diarrhea compared to subjects that were given wild type EPEC (4). While this study was performed using adult volunteers, it nevertheless suggests that BFP are important in the virulence of EPEC infection *in vivo*. However, *in vivo* studies, which imply that BFP production is necessary for both EPEC virulence and eliciting an immune response, still do not identify at what stage of the attachment process BFP might be involved. Speculation regarding the role of BFP later in the infection process includes a phase where EPEC microcolonies disaggregate. In doing so, bacterial spreading to other sites along the intestine is permitted (4, 37). The dispersal phase may result from a BfpF-mediated morphological change in pili structure from thin to thick bundles, thereby creating a larger, three-dimensional microcolony, that can easily dissociate from the intestinal epithelium (1, 4, 37, 55). Although a BFP receptor has not been identified, pre-incubating EPEC with a synthetic LacNAc-BSA conjugate was shown to inhibit EPEC LA to HEp-2 cells and resulted in decreased expression of BfpA (102). This study not only suggests that N-acetyl lactosamine is a potential BFP receptor, but perhaps after BFP-mediated host cell binding, a subsequent decrease in BFP expression could contribute to EPEC dissemination. Thus, EPEC may coordinately down-regulate *bfpA* expression and modify pre-existing BFP into thicker bundles, which would promote spreading and allow EPEC to persist in the host.

During our investigations into the regulation of BFP expression, we observed that growing wild type EPEC in DMEM containing glucose, arabinose, glucosamine, or gluconate, which are carbohydrates that induced *bfpA* expression (Figure 9A), also induced EPEC LA to HEp-2 cells (Figure 10). While my results did not completely reflect *bfpA* promoter expression observed in the *lux* reporter strain E2348/69(pMS420), as we predicted they would, they are however consistent with previous studies indicating that BFP are involved in EPEC LA binding to tissue culture cells. We also observed that when EPEC are grown in DMEM containing galactose, *bfpA* expression is greatly reduced as compared to when the bacteria were grown in the presence of glucose or the other carbohydrates tested (Figure 9). In this case, the lack of *bfpA* expression corresponded with no EPEC binding to HEp-2 cells (Figure 10). Given that EPEC grown in DMEM containing galactose grows much more slowly than when EPEC is grown in DMEM containing glucose, arabinose, glucosamine, or gluconate, the observed *bfpA* expression is likely growth phase-regulated as opposed to being regulated by the carbohydrate source as previously thought (101). There could be a number of reasons why a decreased growth rate has such an extreme effect on *bfpA* expression in EPEC. One possibility is that changes in DNA supercoiling occur such that the BfpTVW regulatory complex can no loner bind to the *bfpA* promoter and activate transcription. Alternatively, the BfpTVW activator complex may not be assembled properly. Even if a *bfpA* transcript is made, the slow growth rate my provide enough time for RNA degradation to take place.

Most interesting, was the observation that E2348/69(pMS420), which contains the *bfpA* promoter in the *lux*-reporter plasmid, expressed *bfpA* but did not bind to HEp-2 cells

(Figures 9B and 10 respectively). TEM results (Figure 12) suggest that perhaps BFP are not assembled properly at the cell surface, which would account for the lack of HEp-2 cell binding exhibited by this strain (Figure 10). Alternatively, BFP may not be the primary adhesin responsible for EPEC LA to tissue culture cells. If this is the case, our results are more consistent with those of Hicks et al. who speculate that BFP are not required for initial adherence to host cells, and that another adhesin awaits recognition (37).

E2348/69(pLux/σ70), which does not contain the cloned *bfpA* promoter neither expressed *bfpA*, nor exhibited LA binding to HEp-2 cells (Figures 9B and 10 respectively). It is difficult to speculate as to why this occurred since E2348/69(pLux/σ70) still possessed the EAF plasmid, and therefore the *bfpA* gene (Dr. Surette, University of Calgary, personal communication). This strain may have suffered a mutation in either the *bfpA* gene, or in one of the genes encoding the BfpTVW regulator complex. DNA hybridization using a *bfpA* gene probe, (29, 73) would confirm the presence of pEAF in E2348/69(pLux/σ70), and northern blot analysis could be performed in order to determine if this strain is making a *bfpA* transcript. pLux/σ70 could also be transformed into another EPEC background rather than E2348/69 to see if this would restore endogenous *bfpA* expression. In E2348/69(pMS420) the presence of the *lux*-reporter plasmid does not hinder endogenous *bfpA* expression (Figure 9B).

Following initial attachment and signaling events between EPEC and the host cell including the production of EspA filaments, Intimin, the outer membrane adhesin, binds to its receptor, Tir, on host cells, facilitating intimate binding and production of A/E lesions (Figure 1). Tir is encoded on the LEE in the EPEC chromosome, and upon synthesis is translocated into the host cell plasma membrane where it serves as the Intimin receptor

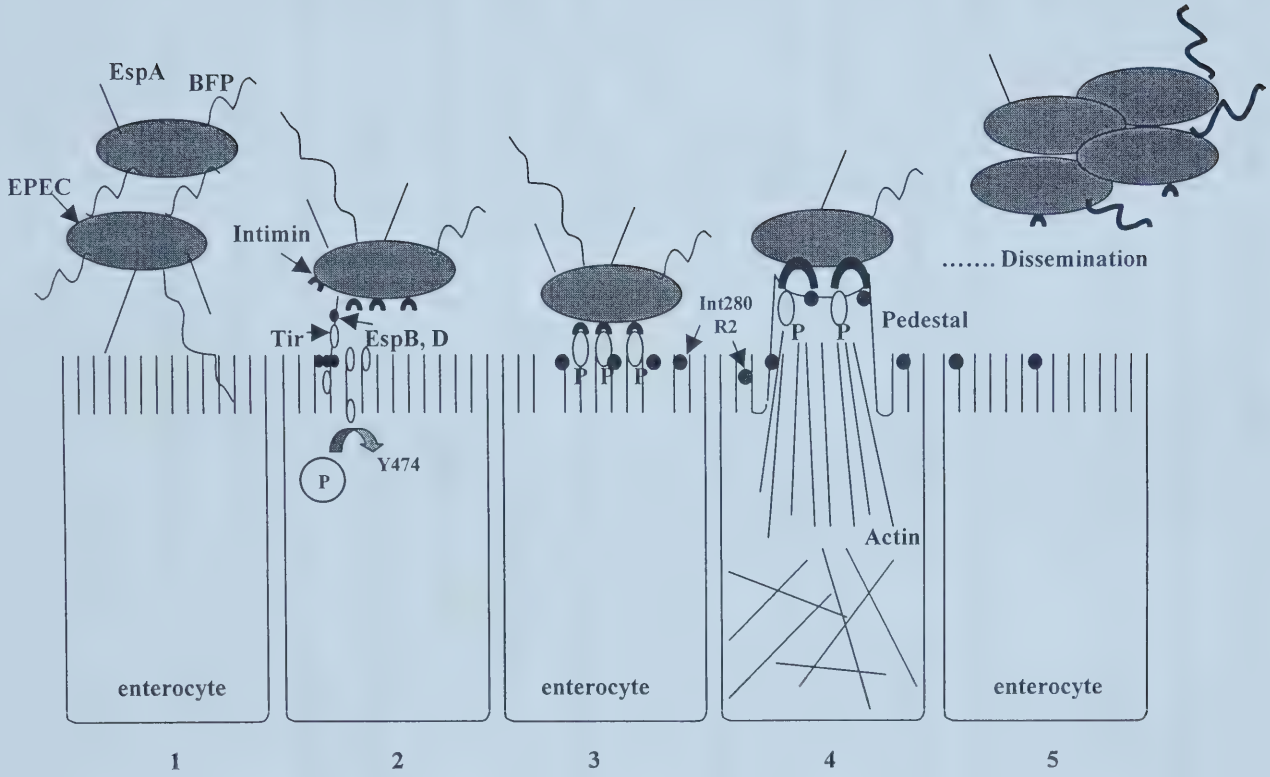
(48). Tir is thought to possess two transmembrane domains, intracellular N and C-termini, and an extracellular loop to which the Intimin ligand binds (9). Upon translocation, the host cell phosphorylates Tir on C-terminal tyrosine residues, but whether or not this event is required for Intimin binding is unclear (9). The cell-binding activity of Intimin has been localized to the C-terminal 280 amino acids (Int280), and purified Intimin binding to mammalian cells in the absence of Tir has been previously demonstrated (21, 24). Arising from this, and the finding that Int280 contains a C-type lectin domain (45), there has been speculation that in addition to Tir, Int280 also recognizes a second receptor of host cell origin. Previously, a synthetic Le^X-BSA glycoconjugate that was pre-incubated with EPEC was shown to slightly decrease Intimin expression (102). This observation together with the knowledge that lectins are a family of proteins that recognize cell surface carbohydrates (107), led us to predict that Int280 possesses a functional carbohydrate recognition domain (CRD) capable of recognizing the eukaryotic surface antigen Le^X. However we were unable to demonstrate binding activity between purified 6xHis-Int280 and LeX-BSA, or other BSA-glycoconjugates in an Elisa assay, or by immunoblotting (Figures 18-20). Our results do not necessarily imply that Int280 does not possess a functional CRD. Perhaps our methods, which are more routinely used to detect protein-protein interactions, were not sensitive enough for detecting protein-carbohydrate interactions, which are typically of low affinity (50). Biacore, a technique that does not require the ELISA washing steps, which inevitably lead to increased dissociation of the ligand from its receptor, may be a superior approach to detecting such low affinity protein-carbohydrate interactions (94, 106). Alternatively, the carbohydrates we tested may bear no resemblance to carbohydrate sequences that may function as Intimin receptors *in vivo*.

While Int280 is similar to C-type lectins, it does not contain the typical residues that bind to calcium, a common feature of carbohydrate binding by C-type lectins (64, 66). Int280 may more closely resemble the tumor necrosis factor stimulated gene-6 (TSG-6) link module, which binds to hyaluronan (45, 57). Regardless, considering that Int280 can bind to host cells in the absence of Tir, resembles C-type lecins, and has been shown to bind to such receptors as β1-Integrins (20), continuing to probe for a second eukaryotic Intimin receptor is rationally warranted. The real question is why Int280 would bind to two receptors, one of EPEC, and the other of eukaryotic origin? Since EPEC employs a sophisticated mechanism for delivering its own receptor (Tir) into host cells, it is reasonable to speculate that Int280 recognizing a host receptor is a secondary event that may serve to stabilize the intimate interaction mediated by Tir-Intimin binding. Many mammalian endothelial cell surface carbohydrates such as Le^x, sialyl-Le^x, and Le^a are recognized by various selectins, a process mediating leukocyte trafficking and initiation of the immune response (34, 61, 103). If EPEC were to gain access to underlying tissues through possible tight junction disruption during the course of infection, Int280 could similarly recognize a carbohydrate receptor. This interaction would then serve to inhibit selectin-dependent leukocyte recruitment, thus allowing EPEC to interfere with the normal inflammation process and avoid host detection. Furthermore, Intimin expression is reduced following A/E lesions (52) and following EPEC pre-incubation with Le^X-BSA (102). Perhaps once intimate attachment and A/E lesions have developed via Tir-Intimin interactions, Int280 binds to a host cell carbohydrate (or other) receptor at which time Intimin expression becomes slightly reduced in preparation for bacterial dissemination to other sites along the intestine. The proposed model of EPEC pathogenesis presented in

Figure 1 may be even more complex than originally thought, and we present a revised model, which may more accurately represent the EPEC infection process (Figure 21).

Figure 21: New proposed model for EPEC infection

EPEC makes initial contact with the host cell through bundle-forming pili (BFP), EspA filaments, or other adhesins (**1**). Upon initial contact effector proteins including EspB and Tir (translocated intimin receptor) are translocated to the host cell (**2**). Tir is phosphorylated on tyrosine and signal transduction events between EPEC and the host cell commence. Intimin binds to phophorylated Tir by means of the C-terminal binding domain (Int280) facilitating intimate binding (**3**). Host cell cytoskeletal rearrangements occur, causing actin to accumulate forming pedestals beneath sites of attached bacteria (**4**). Int280 may also recognize a host cell carbohydrate receptor (Int280 R2) which serves to stabilize the intimately bound EPEC to the host cell and to evade the host immune response. Following A/E lesions, Intimin and BFP expression are reduced and existing BFP may undergo a morphological change from thin to thick bundles (**5**). Larger three dimensional microcolonies result in EPEC lifting away from the enterocyte surface, and the organisms spread to other sites along the intestine.


EspA
BFP
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